# STUDIES ON PATHOGENICITY AND IMMUNOPATHOGENESIS OF INFECTIOUS BRONCHITIS VIRUS INFECTION IN CHICKENS

Thesis submitted in accordance with the requirements of the

**University of Liverpool**

for the degree of

**Doctor in Philosophy**



by

**G. DHINAKAR RAJ**

October, 1996.

**Dedicated**

**to**

**My beloved parents**

# CONTENTS

## Preface

The experimental work described in this thesis was conducted in the Jordan Building, Department of Veterinary Pathology, Leahurst, University of Liverpool, U.K. between 1994 and 1996, during the tenure of a scholarship awarded to the author by the Association of Commonwealth Universities.

## Acknowledgements

I find an ordinary 'thank-you' entirely inadequate to express my sense of gratitude to Dr. R. C. Jones for his inspiring guidance and unstinted help and encouragement during the entire course of this study. However, for want of a superlative degree for this word in the english lexicon, I would still like to say 'car-loads of thank-you' for all his help and most of all, providing freedom in planning and execution of this research work.

I am highly indebted to Mrs. Carol Savage for her invaluable help, both inside and outside of the lab and the keen interest she evinced in the progress of my work.

My thanks are due to Dr. J. K. A. Cook, Intervet, UK for providing me with several IBV isolates including the IBV pool and her useful suggestions. I am also thankful to Dr. T. F. Davison, Institute of Animal Health, Compton for providing the anti-chicken Ig and T-cell monoclonal antibodies and Dr. Brain Adair, Veterinary Sciences Division, Stormont for the Semliki Forest virus.

I am also thankful to Mr. Malcolm Savage for performing the serum creatine kinase and acid phosphatase estimations and help with the calmodulin assay and Mr. Ibrahim Gokce for help with the tumour necrosis factor assay.

I wish to express my heart-felt gratitude to Dr. F. T. W. Jordan, Dr. J. M. Bradbury and Dr. Zerai Woldehiwet for their help and suggestions from time to time. I am thankful to Karen Worthington and Barbara for their help in a variety of ways. Sue and Tony Jopson deserve special thanks for their help in management of isolation units.

My sincere thanks are due to the Association of Comonwealth Universities for their financial support to carry out this work.

Finally, I wish to remember with gratitude, the love and encouragement I received from all members of my family. A special word of thanks is due to my wife for her endurance and my daughter who was a great source of strength and inspiration to me during this period of study.

# Abstract

This thesis describes experimental work to investigate various aspects of pathogenicity and immunopathogenesis of infectious bronchitis virus (IBV) infection in chickens.

Oviduct organ cultures (OOC) were prepared from precocious oviducts induced in young chicks by oestrogen treatment. Using the time taken for a 50% reduction in ciliary activity in OOC and a more sensitive calmodulin assay, seven strains of IBV belonging to different serotypes were grouped in to oviduct pathotypes. Virus titres of six commercial vaccines and five virulent strains of IBV in OOC and tracheal organ cultures (TOC), were measured by three different parameters to determine endpoints viz. ciliostasis, immunofluoresence and organ culture infectivity. It was found that for the two most attenuated vaccines only, the titres in OOC and TOC determined by immunofluoresence were significantly higher than those determined by ciliostasis. Hence, measurement of this difference was proposed to be a useful *in-vitro* marker of attenuation of candidate vaccine viruses. OOC prepared from oestrogen-treated embryos were not as efficacious in supporting the growth of vaccine strains of IBV as those prepared from oestrogen-treated chicks.

Local antibody production in the gut and oviduct of 16-week old hens infected with IBV was demonstrated using comparison of virus-specific IgG and IgA titres in supernatants of explants of selected tissues incubated at 37 $^0$C and 4 $^0$C. It was also shown that IBV-specific antibodies transuded in to the oviducts later in the course of infection. The importance of local immunity in protection of oviducts was investigated using *in-vitro* challenge of OOC prepared from vaccinated or infected chickens. It was found to be less protective compared to that in the trachea. The protectotypic and serotypic classification of five IBV strains (two vaccine and three virulent) were different and protection afforded by an existing vaccine to a heterologous challenge virus was demonstrated. The use of protection of ciliary activity in OOC as an alternative criterion to protection against drop in egg production to evaluate efficacy of IBV vaccines was proposed.

The immunopathogenesis of an economically important variant IBV seen in the UK and Western Europe was examined in day-old specific-pathogen-free (SPF) chicks and six-week old broiler chickens. It was found to be less pneumotropic than enterotropic. However, the spread and seroconversion caused by this virus were similar to other known IBV strains. IgM antibodies were found in the kidneys of infected chickens during the times when virus could not be isolated from this organ. It was suggested that formation and deposition of immune complexes in the capillary walls of muscles might be a contributory factor in muscle changes seen after infection with this variant strain. T-cells were recruited earlier than B-cells in infected tracheas, lungs and kidneys and the virus did not cause immunosuppression.

The role of T-cells in primary and persistent infections of IBV was studied using cyclosporin (CSP) to deplete T-cells *in-vivo*. T-cells protected chickens against IBV-induced mortality and a T-cell suppressed resistant line of chicken behaved like a sensitive line, in terms of mortality. Re-excretion of IBV could not be induced by CSP treatment from chickens infected at two-weeks of age but was possible in chicks infected at day-old. The kidney was found to be the site of virus persistence. Heteropaenia was induced in two-week old chickens and it was found that heterophils were ineffective in neutralising IBV but in fact contributed to the severity of tracheal lesions. The cellular immune responses induced by a live IBV vaccine were found to be cross-reactive to heterologous viruses.

Finally, it was shown that chicken or turkey blood could be stored at 4 $^0$C for only 24 hours without significant reduction in lymphoproliferative responses to mitogens.

# List of publications and presentations

The following publications and presentations have resulted from the work described in this thesis:

## PUBLICATIONS IN REFEREED JOURNALS

DHINAKAR RAJ, G. AND JONES, R. C. (1996) An *in-vitro* comparison of the virulence of seven strains of infectious bronchitis virus using tracheal and oviduct organ cultures. **Avian Pathology** 25: (in press)

DHINAKAR RAJ, G. AND JONES, R. C. (1996) Growth of infectious bronchitis virus vaccines in oviducts derived from oestrogen-treated chicks and embryos. **Vaccine** (in press)

DHINAKAR RAJ, G. AND JONES, R. C. (1996) Local antibody production in the oviduct and gut of hens infected with an enterotropic strain of infectious bronchitis virus. **Veterinary Immunology and Immunopathology** (in press)

DHINAKAR RAJ, G. AND JONES, R. C. (1996) Protectotypic differentiation of avian infectious bronchitis viruses using an *in-vitro* challenge model. **Veterinary Microbiology** (in press)

DHINAKAR RAJ, G. AND JONES, R. C. (1996) Immunopathogenesis of infection in SPF chicks and commercial broilers of a variant infectious bronchitis virus of economic importance. **Avian Pathology** 25: 481 - 501

DHINAKAR RAJ, G. AND JONES, R. C. (1997) Effect of T-cell suppression by cyclosporin on primary and persistent infections of infectious bronchitis virus in chickens. **Avian Pathology** (in press)

DHINAKAR RAJ, G., SAVAGE, C. E. AND JONES, R. C. (1997) Effect of heterophil depletion by 5 fluorouracil on infectious bronchitis virus infection in chickens. **Avian Pathology** (in press)

DHINAKAR RAJ, G., JONES, R. C. AND SAVAGE, C. E. (1996) Effect of storage of chicken and turkey blood on the lymphocyte responses to concanavalin A and pokeweed mitogen. **Veterinary Immunology and Immunopathology** (in press)

## OTHER PRESENTATIONS

DHINAKAR RAJ, G., SAVAGE, C. E. AND JONES, R. C. (1995) *In-vitro* pathogenicity and tropism of infectious bronchitis virus isolates for the chicken oviduct. Paper presented to the Annual Meeting of the Association of Veterinary Teachers and Research Workers, Scarborough, 11 - 13 April, 1995

DHINAKAR RAJ, G., SAVAGE, C. E. AND JONES, R. C. (1995) Early immunopathogenesis in chicks of an economically important variant infectious bronchitis virus in United Kingdom. Paper presented to the Annual Meeting of the Association of Veterinary Teachers and Research Workers, Scarborough, 11 - 13 April, 1995

DHINAKAR RAJ, G. AND JONES, R. C. (1995) Immunopathogenesis of an isolate of IBV variant 793/B. Paper presented to The British Veterinary Poultry Association Spring Meeting, Harrogate, 4 - 6 April, 1995

DHINAKAR RAJ, G. AND JONES, R. C. (1995) Infectious bronchitis viruses: pathotypes and protectotypes. Paper presented to The British Veterinary Poultry Association Winter Meeting, Harrogate, 29 -30 November, 1995

DHINAKAR RAJ, G. AND JONES, R. C. (1996) Preliminary studies on local antibody production in the oviduct and gut of hens infected with a variant strain of infectious bronchitis virus.(poster abstract) In Poultry Immunology, Poultry Science Symposium series vol. 24. Eds. T. F. Davison, T. R. Morris and L. N. Payne pp 446. Carfax Publishing Company, Abingdon, England.

DHINAKAR RAJ, G. AND JONES, R. C. (1996) The role of T-cells in infection of chickens with infectious bronchitis virus. Paper presented to The British Veterinary Poultry Association Spring Meeting, Scarborough, 19 - 21 March, 1996

DHINAKAR RAJ, G. AND JONES, R. C. (1996) The role of T-cells in infectious bronchitis in chickens. Paper presented to the Annual Meeting of the Association of Veterinary Teachers and Research Workers, Scarborough, 2 - 4 April, 1996

JONES, R. C. AND DHINAKAR RAJ, G. (1996) Development of attenuated avian respiratory virus vaccines: problems and possible solutions. Keynote invited paper presented at the XX World's Poultry Congress, New Delhi, 2-5 September, 1996. Proceedings volume II pp 443 - 454

## Acronyms and abbreviations used

| | |
|---|---|
| BLH | brown leghorn (chickens) |
| BSA | bovine serum albumin |
| $^0C$ | degree celsius |
| CAM | calmodulin |
| cAMP | adenosine 3', 5'-cyclic monophosphate |
| CD | clusters of differentiation |
| $CD_{50}$ | 50% ciliostatic dose |
| CEF | chicken embryo fibroblast |
| Ci | curie |
| CIT | cross-immunity test |
| CK | creatine kinase |
| cm | centimetre |
| CMI | cell-mediated immunity |
| $CO_2$ | carbon dioxide |
| Con A | concanavalin A |
| CPE | cytopathic effect |
| cpm | counts per minute |
| CSP | cyclosporin |
| ECE | embryonated chicken eggs |
| EDTA | ethylene diamine tetra acetate |
| ELISA | enzyme-linked immunosorbent assay |
| FCS | foetal calf serum |
| 5 FU | 5-fluorouracil |
| g | gramme |
| HA | haemagglutination |
| HG | Harderian gland |
| HI | haemagglutination-inhibition |
| HEPES | 2-hydroxy-ethyl-piperazine-N'2-ethane sulphonic acid |
| IB | infectious bronchitis |

| | |
|---|---|
| IBV | infectious bronchitis virus |
| IF | immunofluoresence |
| $IFID_{50}$ | 50% immunofluoresence infective dose |
| γ-IFN | gamma-interferon |
| Ig | immunoglobulin |
| IL-1 | interleukin-1 |
| IL-2 | interleukin-2 |
| IP | immunoperoxidase |
| LT | lymphocyte transformation |
| μ | micron |
| M | molar concentration |
| Mab | monoclonal antibody |
| 2 ME | 2 mercaptoethanol |
| MEM | minimum essential medium |
| μg | microgramme |
| mg | milligramme |
| min | minutes |
| ml | millilitre |
| μl | microlitre |
| mM | milli-molar concentration |
| MNC | mononuclear cells |
| mRNA | messenger ribonucleic acid |
| MTT | 3-(5, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide |
| n | number of samples |
| N | nucleocapsid protein of IBV |
| NDV | Newcastle disease virus |
| NIBV | nephropathogenic infectious bronchitis virus |
| nm | nanometre |
| $OCID_{50}$ | 50% organ culture infective dose |
| OD | optical density |
| OOC | oviduct organ culture |
| OPD | O-phenylene diamine |
| p | probability that null hypothesis is valid |
| PBS | phosphate buffered saline |

| | |
|---|---|
| PBST | phosphate buffered saline-tween-20 |
| p.i. | post-infection or post-inoculation |
| PHA | phytohaemagglutinin |
| PPCA | percent peripheral ciliary acitivity |
| PWM | pokeweed mitogen |
| p.v. | post-vaccination |
| | |
| RBC | red blood cells |
| RCA | relative ciliary activity |
| rpm | revolutions per minute |
| RPMI | Roswell Park Memorial Institute media |
| | |
| S | spike protein of IBV |
| sd | standard deviation |
| SDS | sodium dodecyl sulphate |
| SEM | standard error of mean |
| SI | stimulation index |
| SN | serum neutralisation |
| SPF | specific-pathogen-free |
| | |
| $TCID_{50}$ | 50% tissue culture infective dose |
| TNF | tumour necrosis factor |
| TNLF | tumour necrosis like-factor |
| TOC | tracheal organ culture |
| Tris | (hydroxy methyl) aminomethane |
| TRTV | turkey rhinotracheitis virus |
| Tween | polyoxyethylene sorbitan moolaurate |
| | |
| UK | United Kingdom |
| USA | United States of America |
| | |
| v / v | volume / volume |
| VN | virus neutralisation |
| | |
| WBC | white blood cells |
| WLH | white leghorn (chickens) |
| w / v | weight / volume |

# CHAPTER 1

## INTRODUCTION AND AIMS

A 'new respiratory disease of baby chickens' was identified in the USA by Schalk and Hawn in 1931 and named infectious bronchitis (IB). The causative virus, infectious bronchitis virus (IBV) was isolated by Beaudette and Hudson (1937) and its characteristic coronavirus morphology described by Berry et al. (1964). Since then IBV has been considered to be the prototype virus of the Family Coronaviridae. Other coronaviruses and their host species include, human: human coronavirus 229E and OC43; swine: transmissible gastroenteritis virus and the respiratory variant, porcine respiratory coronavirus, porcine epidemic diarrhoea virus and haemagglutinating encephalomyelitis virus; cattle: bovine coronavirus; horses: equine coronavirus; turkeys: turkey coronavirus; dogs: canine coronavirus; cats: feline infectious peritonitis virus and feline enteric coronavirus; mouse: mouse hepatitis virus; rats: sialodacryadenitis virus and rabbits: rabbit coronavirus (Holmes, 1990; Saif, 1993).

IB is considered primarily a respiratory disease of chickens, but other disease manifestations such as decline in egg production and quality, kidney damage, enteritis and even pectoral myopathy have been observed. The economic importance of the disease is due to poor weight gain and feed efficiency, decline in egg production and quality, increased number of eggs unacceptable for setting, reduced hatchability, mortalities and increased condemnation at slaughter due to airsacculitis caused by mixed infections involving IBV and bacteria or mycoplasmas.

The disease has been effectively controlled by the use of vaccines. However, the constant emergence of new antigenically-distinct variants, their wide and varied tissue tropism and lack of a broad-spectrum vaccine capable of protecting against all serotypes has hampered the control programmes to the extent that IB is still a major economic problem some 65 years after it was first reported.

The aim of the work described in this thesis was to study certain aspects of pathogenicity and immunopathogenesis of IBV infection in chickens. Major areas which have been studied are :

(1) The virulence of seven IBV strains for oviduct organ cultures (OOC), prepared from precociously-induced oviducts from young chickens after oestrogen treatment, was compared in an attempt to classify these strains in to oviduct pathotypes.

(2) The growth of vaccine strains of IBV was compared in OOC prepared from oestrogen-treated chickens or embryos. The difference in virus titres determined by ciliostasis or immunofluorescence as endpoints, was proposed for use as an *in-vitro* attenuation marker for IBV.

(3) A study was carried out on local antibody production in the oviduct and gut of IBV-infected hens.

(4) The importance of local antibodies in the oviducts was assessed, in terms of protection of OOC against direct damage caused by the challenge virus. The serotypic and protectotypic differentiation of IBV strains at the tracheal and oviduct levels was done using an *in-vitro* challenge model.

(5) The immunopathogenesis of the currently most economically important recent IBV variant in the UK, strain 793/B, was studied in day-old chickens and six-week old broiler chickens.

(6) The role of T-cells in primary and persistent infections of IBV was studied using cyclosporin (CSP) to deplete T-cells *in-vivo*.

(7) The role of heterophils in virus-infections of poultry is unknown. An attempt was made to investigate the importance of these cells in IBV infections using a heteropaenic chicken model.

(8) The *in-vivo* protection afforded by IBV vaccines often crosses the serological barrier. To try to account for this phenomenon, cross-reactive cellular immune responses induced by a live IBV vaccine were analysed.

(9) The effect of storage of whole blood of chickens and turkeys on the mitogen responses of lymphocytes was studied.

# CHAPTER 2

## REVIEW OF LITERATURE

**CONTENTS** **PAGE NUMBER**

# CHAPTER 2

## REVIEW OF LITERATURE

### 2.1. INFECTIOUS BRONCHITIS - THE DISEASE

#### 2.1.1. Natural hosts

The domestic chicken has been regarded as the exclusive host for IBV. However, IBV has also been isolated from pheasants in association with respiratory disease, egg production and shell quality problems (Spackman and Cameron, 1983) or nephritis (Gough et al., 1996) and racing pigeons with respiratory symptoms (Barr et al., 1988). IBV antibodies have also been demonstrated in the serum of ostriches (Cadman et al., 1994).

#### 2.1.2. Transmission

IBV spreads rapidly among chickens in a flock with in-contact susceptible chickens developing signs within 48 hours. The most common route of transmission is air-borne. Other routes of spread include infected faeces and fomites (Purchase et al., 1966; Cook, 1968; Alexander and Gough, 1977). Vertical transmission has been reported (Cook, 1971) but is considered to be of little importance. Virus may persist for extended periods in the bird and may be re-excreted in response to laying stress (Jones and Ambali, 1987) or T-cell suppression by CSP (Bhattacharjee et al., 1995). Extended and intermittent shedding of virus may serve as a potential risk for flock-to-flock transmission. Vectors are not known to be involved in the transmission of IBV.

#### 2.1.3. Geographical distribution

IB is distributed world-wide and variant serotypes of the virus continue to be isolated even from vaccinated flocks. Some new variants of the virus arise and disappear without causing significant economic losses or warranting the production of new vaccines (Cook, 1984; Cook and Huggins; 1986). Others persist causing significant economic losses (Cook et al., 1996) and new vaccines have been prepared to control such variants.

There is considerable geographic variation in the virulence and tropism of IBV. Nephropathogenic IBV (NIBV) was first reported in Australia (Cumming, 1962) and subsequently from USA and parts of Europe. However, it appears to be limited to a restricted area in Europe (Lambrechts et al., 1993) unlike the more recent variant IBV in UK (Gough et al., 1992; Parsons et al., 1992) which has been shown to be prevalent

even in Mexico and Thailand (Cook et al., 1996). Both these strains differ in the S1 amino acid sequences from the commonly used vaccine strains by more than 20% (Adzhar et al., 1995; Shaw et al., 1996). However, the apparent reason for the differences in geographical distribution of these two strains is not known but may be related to the severity of disease they cause (Cook et al., 1996).

### 2.1.4. Clinical signs

The incubation period for the disease is normally 18 - 36 hrs. All the birds in a flock become infected but in uncomplicated cases mortalities are generally low. The affected chickens appear depressed and feed consumption and weight gain are significantly reduced from 3 days after infection (Otsuki et al., 1990). The characteristic respiratory signs of IBV are gasping, coughing, tracheal rales and nasal discharge. Occasionally, puffy, inflamed eyes and swollen sinuses may be seen (Parsons et al., 1992; Capua et al., 1994). In uncomplicated cases respiratory signs last for only 5 - 7 days and disappear with in 10 - 14 days.

In layers, decline in egg production, egg size and internal quality are seen (Sevoian and Levine, 1957; McDougall, 1968), with mild (Muneer et al., 1986; Muneer et al., 1988) or no respiratory signs (Cook, 1984; Cook and Huggins, 1986). Some strains produce only a loss in shell colour (Cook and Huggins, 1986). The severity of production decline varies with the period of lay and the virulence of the virus involved. The response of individual hens varies greatly (McMartin, 1968; Jones and Jordan, 1972). Production may start to increase after 2 - 3 weeks but reaches only sub-optimal levels. When laying is resumed, eggs show various abnormalities like soft-shell, mis-shapen or rough-shell eggs. Internal quality is also affected with the albumen being thin and watery. Presence of blood or meat spots in the egg albumen has been reported (McDougall, 1968; Muneer et al., 1987)

NIBV initially causes respiratory symptoms followed by signs due to kidney damage which include increased water consumption and wet droppings (Winterfield and Hitchner, 1962; Cumming, 1963). Mortalities occur and follow a consistent pattern (Cumming and Chubb, 1988). The first deaths occur around 6 days, increase rapidly to a peak around 10 days with the last deaths seen around 16 days. However, the mortality rates depend on several intrinsic (age and breed of the birds) and extrinsic (environment and nutrition) factors (see below).

Swollen head syndrome (SHS) is a condition where chickens have swollen heads due to fascial cellulitis and swollen eyelids. Although SHS has been associated with a pneumovirus, turkey rhinotracheitis virus (TRTV) (Picault et al., 1987; Jones et al.,

1991), a Massachusetts strain of IBV has also been isolated from chickens with SHS (Shirai et al., 1993; Droual and Woolcock, 1994) and a coronavirus was isolated by Morley and Thomson (1984) in the first description of SHS. The role of IBV in the pathogenesis of SHS is not certain although it has been postulated that *Escherichia coli* may invade the air spaces of the cranial bones through the eustachian tube following an upper respiratory tract viral infection (Droual and Woolcock, 1994).

A new variant strain of IBV isolated in the UK, variably designated as 793/B or 4/91 (Gough et al., 1992; Parsons et al., 1992), has been shown to cause variable mortality in broiler breeders. The affected birds had cyanosed combs with hyperventilation and muscle tremors seen before rapid prostration and death.

### 2.1.5. Pathology

#### 2.1.5.1. Gross changes

Infected chickens have mucosal thickening with serous or catarrhal exudate in the nasal passages, sinuses and trachea. The incidence of nasal exudate in infected chickens has been used to assess the severity of disease in different lines of chickens (Parsons et al., 1992) and in birds devoid of the bursa (Cook et al., 1991a). Caseous casts may be seen blocking the lower trachea or bronchi which may result in the death of the bird by asphyxiation. Small areas of pneumonia may be observed in the lungs. The air sacs may appear cloudy or contain a yellow caseous exudate (King and Cavanagh, 1991).

Inspissated yolk material may be seen in the abdominal cavity of infected layers. Following early infection of chickens, gross changes in the oviduct may vary from the presence of a continous patent but underdeveloped structure to a blind sac projecting forward from the cloaca (Jones and Jordan, 1970; Jones and Jordan, 1972). The middle third of the oviduct is the most severely affected with areas of localised hypoplasia seen in-between normal patent oviducts. Caudal to the hypoplastic regions macroscopic cysts filled with a clear serous fluid may be seen (Crinion et al., 1971a).

The kidneys of chickens infected with NIBV are swollen and pale with tubules and ureters distended with urates (Cumming, 1963). The relative kidney weight and kidney assymetry are increased. The haematocrit values of infected birds was decreased and plasma uric acid levels were increased (Afanador and Roberts, 1994). Despite lack of gross lesions microscopic changes of nephritis may still be present (Winterfield and Albassam, 1984).

The new variant strain of IBV, 793/B, was recently isolated from a broiler breeder flock where the affected birds had bilateral myopathy affecting both deep and superficial pectoral muscles (Gough et al., 1992). There was marked swelling and pallor of deep pectoral muscles together with the presence of occasional fascial heamorrhages and a layer of gelatinous oedema over its surface. A study of the immunopathogenesis of this variant virus infection in day-old and six-week old broiler chickens has been done in Chapter 8.

### 2.1.5.2. Histopathology

The histopathological changes are not pathognomonic for IBV but is useful in experimental conditions for assessing the severity of the disease (Ratanasethakul and Cumming, 1983a; Davelaar and Vandenboss, 1992; Nakamura et al., 1991) and to measure protection afforded by vaccines (Andrade et al., 1982; Yachida et al., 1985; Klieve and Cumming, 1990).

The progression of lesions in the trachea can be divided into three stages, degenerative, hyperplastic and recovery (Purcell and McFerran, 1972; Nakamura et al., 1991). Deciliation and desquamation of epithelial and mucous-secreting cells are noticed in the first 1-2 days with a mild infiltration of heterophils and lymphocytes in the lamina propria. Heterophils are often seen infiltrating between ciliated epithelial cells and occasionally in the lumen of the trachea. By 4-6 days reparative processes begin with complete recovery by 10 - 20 days (Chen et al., 1996). IBV damaged both the upper and lower respiratory tract unlike infectious laryngotracheitis virus which primarily affected the upper respiratory tract (Nakamura et al., 1996). Epithelial cell desquamation, oedema and some fibrinous exudate may be seen in affected airsacs (King and Cavanagh, 1991).

The changes in the oviduct include decreased height and loss of cilia from epithelial cells, dilation of the tubular glands, infiltration of heterophils, lymphocytes and plasma cells and oedema and fibroplasia of the lamina propria (Sevoian and Levine, 1957; Crinion et al., 1971a,b; Crinion and Hofstad, 1972a,b).

Chen et al. (1996) proposed that IBV-induced renal lesions can be considered to be a ductotubular interstitial nephritis. The virus causes granular degeneration, vacuolation and desquamation of the tubular epithelium with massive infiltration of heterophils in the interstitium in acute stages of the disease. The changes in the chronic phase was classified as being active or inactive types of interstitial lymphocytic nephritis (Albassam et al., 1986). The chronic inactive form of nephritis was indicative of IBV replication in the kidneys and subsequent clearance; while the chronic active type

suggested a persistent viral replication and damage to epithelial cells of the kidney tubules. Virus was isolated from 50% of the birds with chronic nephritis (Chong and Apostolov, 1982). The histopathological changes in the kidneys following NIBV infections have been described by several workers (Siller and Cumming, 1974; Pohl, 1974; Purcell et al., 1976; Albassam et al., 1986; Chen et al., 1996).

In the Harderian gland of chickens, IBV vaccination by eyedrop resulted in a massive infiltration of lymphocytes, increase in plasma cell numbers (Survashe et al., 1979; Davelaar and Kouwenhoven, 1976; Montgomery et al., 1994; Toro et al., 1996) and desquamation of tubular epithelium with restoration from 14 days after vaccination (Toro et al., 1996). Increased numbers of plasma cells (Survashe et al., 1979) and lymphoid tissue development (Montgomery et al., 1994) were also seen in the lachrymal gland following IBV vaccination.

No histopathological changes have been described in the gut of IBV-infected birds.

## 2.2. PATHOGENESIS

The trachea is the main portal of entry and site of virus replication, following which a viraemia occurs and the virus gets widely disseminated to other tissues. The virus is primarily epitheliotropic and enters the epithelial cells by viropexis (Patterson and Bingham, 1976). Studies using immunofluorescence (IF) (Jones and Jordan, 1972; Yagyu and Ohta, 1990), immunoperoxidase (IP) (Owen et al., 1991; Nakamura et al., 1991) and electron microscopy (Purcell and Clarke, 1972; Nakamura et al., 1991) have shown virus replication in ciliated epithelial and mucus-secreting cells. High virus titres were recorded for 5 - 7 days. From the respiratory system the virus becomes blood-borne within 1 -2 days of infection and is spread to many organs, especially the kidneys, oviduct and gut.

Virus replication in the epithelium of the oviducts in young chicks (Crinion and Hofstad, 1972a,b) and in laying hens (Jones and Jordan, 1971) has been shown. *In-vitro* OOC were highly susceptible to the H52 strain of IBV regardless of the age of the chickens and no differences in susceptibility were seen between magnum and uterus regions (Peters et al., 1979). However, if young chicks are infected, the damage to the oviducts was found to be more severe and permanent (Crinion et al., 1971a; Jones and Jordan, 1972), because of the un-differentiated nature of their oviducts. Areas of glandular hypoplasia caused by IBV leads to reduction in the synthesis of albumen proteins especially ovomucin, lysozyme and other major proteins which constitute the structural matrix of the thick albumen (Butler et al., 1972a). Hence there is a decrease in

the proportion of both thick and inner thin albumen associated with a large increase in the amount of outer thin albumen causing egg quality problems like watery-whites.

Virus replication in the kidneys has been shown in the proximal convoluted tubules (Chong and Apostolov, 1982), distal convoluted and collecting tubules (Owen et al., 1991) and collecting ducts (Chen et al., 1996). Structural alterations in the tubular epithelial cells (Condron and Marshall, 1986) caused impaired fluid and electrolyte transport leading to acute renal failure. An increase in urinary water losses in chickens infected with NIBV was associated with lower urine osmolarity and higher rates of urinary excretion of sodium, potassium and calcium (Heath, 1970; Condron and Marshall, 1985; Afanador and Roberts, 1994). Negative sodium balance was a direct effect of increased output of sodium in the urine while negative potassium balance was due to decreased intake.

The role of the digestive system as a portal of virus entry is uncertain. However, several strains of IBV have been isolated from cloacal swabs, faeces and caecal tonsils (Alexander and Gough, 1977; Alexander et al., 1978; Cook, 1984; Lucio and Fabricant, 1990). *In-vitro* explants of the gut tissues have been shown to support the growth of IBV (Darbyshire et al., 1976; Darbyshire et al., 1978; Bhattacharjee, 1994). Maximum virus isolations were obtained from the oesophagus of chickens infected with ECV2, an enteric isolate of IBV (Lucio and Fabricant, 1990). Oesophageal swabs have also been used to identify IBV by polymerase chain reactions (D. Cavanagh, personal communication). However, it is not clear whether the virus actually multiplies in the oesophagus or whether virus is expelled by coughing from the trachea. IBV has also been isolated from proventriculus, duodenum and jejunum (Ambali and Jones, 1990; Lucio and Fabricant, 1990). Darbyshire et al. (1976) have shown that proventriculus was inferior only to respiratory tissues and oviduct in supporting virus multiplication *in-vitro*. However, the site of virus multiplication in these tissues is not known.

IBV has been shown to multiply in cells resembling histiocytes and lymphoid cells in the caecal tonsils (Owen et al., 1991) and in epithelial cells of the villi in ileum and rectum (Ambali and Jones, 1990). Although IBV has a wide tropism for gut tissues no gross or histological changes have been reported in these tissues.

IBV has also been isolated from the bursa of Fabricius (Ambali and Jones, 1990) and gross and histopathological lesions have been shown following experimental H52 and H120 infections (MacDonald and McMartin, 1976). Virus isolations from Harderian gland have been reported (Gelb et al., 1991; Toro et al., 1996) and IBV- positive cells have been shown by IF in the stroma of the gland (Toro et al., 1996). It is

not clear whether IBV multiplies in the lymphocytes but cultured macrophages were found to be resisitant (von Bulow and Klasen, 1983). A virulent IBV has been shown to induce transitory reduction in proliferative responses of whole blood lymphocytes to a T-cell mitogen, phytohaemagglutin (PHA) (Wakenell et al., 1995). IBV vaccines were found to depress the Harderian gland responses to killed *Brucella abortus* (Montgomery et al., 1991; Montgomery et al., 1994). But no correlation between histological responses and IB haemagglutination-inhibition (HI) titres were found with depression of responses to *Brucella abortus*.

IBV has also been isolated from a variety of other tissues such as lungs, air sacs and spleen (Otsuki et al., 1990), liver (Ambali and Jones, 1990), semen and eggs (Cook, 1971).

### 2.2.1. Influencing factors

Several intrinsic (age and breed of chickens) and extrinsic (nutrition and environment) factors influence the pathogenesis of the disease.

#### 2.2.1.1. Age

All ages are susceptible but the clinical disease was more severe in young chicks (Animas et al., 1994a) although recovery of the virus from the trachea was similar (Animas et al., 1994b). However, the duration of virus excretion in the faeces was longer in 2-week old infected chicks compared to 4- and 6-week old chickens. As age increases chicks become more resistant to IBV-induced mortality (Smith et al., 1985), nephropathic effects (Albassam et al., 1986) and oviduct lesions (Crinion and Hofstad, 1972a). MacDonald et al. (1980) described less pathological changes in kidneys of chickens infected at 3 weeks of age than those infected at day-old or 10 weeks of age. However, the virus was inoculated intravenously (i/v), which is an unnatural route of infection.

#### 2.2.1.2. Breed

Bumstead et al. (1989) compared mortalities in several inbred lines of chickens following inoculation with a pool of strains of IBV and/or *E. coli* and found marked differences among them. On this basis, these lines of chickens were classified as being resistant or sensitive to IBV. However, these inbred lines did not show marked differences in susceptibility to a recent variant strain of IBV in UK (Parsons et al., 1992).

Genetic differences in susceptibility to nephritis were also marked (Cumming and Chubb, 1988). The mortalities in 4-week old cockerels of 10 Australian commercial

egg-laying strains varied from 11 - 59%. NIBV has been shown to cause higher mortalities in broilers than layers (Ignjatovic, 1988; Zanella, 1988) although the virus multiplies to the same extent in both types of birds (Lambrechts et al., 1993). This difference in the clinical outcome of the disease, has been attributed to the higher feed conversion rate in broilers leading to residual nitrogen products being eliminated from the kidneys. Male chicks are twice as susceptible as females to nephritis (Cumming, 1969).

#### 2.2.1.3. Nutritional factors

High protein diets increase mortality from nephrosis; chickens fed meat meal or poultry by-product meal-based diets experience higher mortality than those fed soybean based diets (Cumming, 1969; Cumming and Chubb, 1988).

#### 2.2.1.4. Environmental factors

Low temperatures have a dramatic effect on mortality due to NIBV. Reduction in temperature from $20^0$C to $16^0$C increased mortality from 8 to 50% (Cumming, 1969) with increased severity of histopathological lesions in kidneys (Ratanasethakul and Cumming, 1983b). Exposure to cold stress has been used to increase the severity of challenge for assessing protection afforded by IBV vaccines (Klieve and Cumming, 1990). Cold stress also increased the severity of IBV-induced tracheal lesions (Ratanasethakul and Cumming, 1983b) and promoted more extensive air sacculitis after combined infections with IBV and *Mycoplasma synoviae* (Yoder et al., 1977).

#### 2.2.1.5. Intercurrent infections

IBV infections may not occur as a single entity in the field. With the presence of several respiratory diseases in chickens caused by bacteria and viruses, the role of these agents in increasing the severity of IBV or otherwise is an important contributory factor in influencing the pathogenesis of the disease.

IBV infection of the respiratory tract and subsequent damage of the epithelial lining facilitates *E.coli* invasion resulting in septicaemic lesions and mortality (Smith et al., 1985; Nakamura et al., 1992). Hence mixed infections of IBV and *E.coli* has been used in experimental studies for the production of a severe respiratory disease closely resembling field disease (Smith et al., 1985; Cook et al., 1986; Bumstead et al., 1989; Cook et al., 1991b; Cubillos et al., 1991; Avellanda et al., 1994). Dual infections with IBV and mycoplasmas caused tracheitis and air sacculitis (Adler et al., 1962; Hopkins and Yoder, 1982). Triple infections with IBV or IBV vaccines, Mycoplasma and *E.coli* produced the most severe septicaemic lesions while single infections did not (Fabricant

and Levine, 1962; Nakamura et al., 1994). Aerosol vaccination with IBV increased the mortalities due to infectious laryngotracheitis (Pattison et al., 1971).

## 2.3. INFECTIOUS BRONCHITIS VIRUS

### 2.3.1. Morphology

IBV is the prototype virus of the Family Coronaviradae. These viruses possess an array of outer projections called spikes or peplomers which resemble a corona or crown, hence the name. IBV is generally rounded or pleomorphic with diameters ranging from 80 - 120 nm (Berry et al., 1964). The virion has characteristic pear-shaped spikes approximately 20nm long and 10nm wide (McIntosh et al., 1967) connected to the envelope by the narrow end.

### 2.3.2. Physico-chemical characteristics

Most IBV strains are inactivated at $56^0$C for 15 minutes but sensitivity to heat differs between strains and their passage levels in embryos (Cunningham, 1970; Otsuki et al., 1979a; El Houadfi et al., 1986). Although the virus is ether-sensitive, the treatment may not completely inactivate the infectivity (Otsuki et al., 1979a). The sensitivity to trypsin and bile salts may vary with the strain of IBV (Ambali and Jones, 1991a); M41 was sensitive while an enterotropic variant 'G' was not. Optimum stability is at pH 7.8 but survival between pH 2.0 and 9.0 has been shown. Survival of IBV in water was aided by powdered skim milk, poultry mash and poultry droppings, probably by providing an organic detoxifying substrate (Jordan and Nassar, 1973).

### 2.3.3. Viral genome and polypeptides

IBV has a positive-stranded RNA genome of 27 kilobases which is polyadenylated at the 3' end. In infected cells six mRNA species (A - F) are produced; mRNA 'A' encoded the nucleocapsid protein, mRNA 'C' the membrane protein and mRNA 'E' the spike protein (Binns, 1988). The spike 'S' glycoprotein located at the surface of the virion, consists of two subunits, S1 and S2 with molecular weights of 92K and 84K respectively. The membrane 'M' glycoprotein is partially exposed at the surface of the virion with molecular weight ranging from 27K to 36K and the nucleocapsid 'N' protein is internally located with a molecular weight of 52K (Wadey and Westaway, 1981; Cavanagh, 1983a; Cavanagh, 1983b; Cavanagh, 1983c).

### 2.3.4. Antigenic types (serotypes)

IBV does not constitute a single homogenous antigenic type. The prototype virus is Massachusetts M41. Since the first identification of a different serotype (named

Connecticut) by Jungherr et al. (1956) several new antigenic types have been reported (Cook, 1983; Cook, 1984; Picault et al., 1986; Gelb et al., 1991; Parsons et al., 1992; Capua et al., 1994) in various countries. The major virus neutralising (VN) antibody site of IBV, which defines serotype, resides in the S1 subunit of the spike protein (Mockett et al., 1984; Cavanagh et al., 1986). Differences in only a few amino acids in the S1 protein can result in different VN serotypes (Cavanagh et al., 1992a) which accounts for the plethora of IBV strains which exists today. Hence different antigenic types identified by VN tests does not imply that the isolates have substantially different S1 proteins or overall antigenic properties or greatly different evolutionary lineages.

As in other RNA viruses, antigenic variation is probably facilitated by the high error rate during the transcription of RNA template and the absence of proof-reading mechanism. It has been shown that point mutations may lead to the generation of IBV variants in the field (Jia et al., 1995). But both circumstantial (Cavanagh and Davis, 1988; Kusters et al., 1989; Kusters et al., 1990; Cavanagh et al., 1992b; Wang et al., 1994; Jia et al., 1995) and experimental (Kottier et al., 1995) evidence suggests that the main mechanism of generation of variant strains of IBV is by recombination. This could have been facilitated by the use of more than one strain of IBV for vaccination.

### 2.3.5. Immunogenic types (Protectotypes)

Isolates of IBV shown to be distinct by the VN test can still induce partial or complete cross-immunity (Hitchner et al., 1964; Raggi and Lee, 1965; Winterfield and Fadly, 1972; Winterfield et al., 1976; Darbyshire, 1980; Darbyshire, 1985; Arvidson et al., 1990). For example, vaccination with H120 caused a 30,000-fold reduction in titres of the challenge virus, Australian 'T' strain (Darbyshire, 1985), while on the basis of VN tests *in-vitro*, no evidence of a serological relationship between these two viruses could be demonstrated (Darbyshire et al., 1979). Hence antigenic studies alone do not adequately define immunological relationships between strains. Thus it was suggested that cross-immunisation studies could be used to classify IBV isolates into protectotypes (Lohr, 1988) as this would reduce the large number of serotypes to a smaller number of protectotypes and provide more pratical information to the field.

Cross-immunisation tests have been performed in experimental chickens (Darbyshire, 1980; Darbyshire, 1985; Lambrechts et al., 1993) or in *in-vitro* conditions using tracheal organ cultures (TOC) from immunised birds (Hinze et al., 1991) to determine immunological relationships between IBV strains. Arvidson et al. (1990) have described a model to study immunogenic relationships between IBV strains based on the vaccinating dose required to prevent the multiplication of a standard challenge dose of a homologous strain in the lungs of chickens. These cross-immunisation tests would

help to determine whether an already existing vaccine could offer protection to a new variant. This information is critical before embarking on a long and cumbersome process of a new vaccine production.

### 2.3.6. Pathogenic types (pathotypes)

IBV strains vary widely in tropism and virulence; hence classification has not been attempted using this criterion although NIBV has been well recognised.

Differences in the capacity of various strains of IBV to exacerbate *Mycoplasma synoviae* air sacculitis have been shown (Hopkins and Yoder, 1982). Smith et al. (1985), using a combined inoculation of a pool of strains of IBV and *E. coli*, developed an experimental model for IBV which closely resembled natural outbreaks of the disease. Using this model, differences in virulence of IBV strains has been demonstrated (Cook et al., 1986; Avellanda et al., 1994). Otsuki et al. (1990) found that following infection with M41 strain of IBV, the duration and severity of tracheal ciliary damage was longer in the susceptible line of chicken (15I) than in the resistant line (C). However this method has not been used to compare the pathogenicity of different strains.

Cubillos et al. (1991) found that in unvaccinated chickens challenged with IBV isolates from Chile, the tracheal damage in terms of ciliary activity was variable. Cook et al. (1976), while standardising TOC for the isolation and assay of IBV, compared three strains of IBV on the basis of their effect on tracheal cilia but found no marked differences. Cubillos et al. (1991) compared the histopathological changes in TOC following inoculation with field isolates of IBV and found no marked differences except that hyperplasia was observed only after inoculation with two isolates.

Differences in virulence of IBV strains for the chicken oviduct was reported by Crinion and Hofstad (1972a); Massachusetts and T strains were virulent while Connecticut and Iowa 609 were not. Embryo passage of IBV strain M41 reduced its virulence for the oviduct (Crinion and Hofstad, 1972b). Pradhan et al. (1984) showed that IBV strain M41 causes stasis of cilia in OOC prepared from precociously-induced oviducts in young chicks by oestrogen treatment. This work was extrapolated to compare the virulence of seven strains of IBV *in-vitro* using ciliostasis and a calmodulin assay to quantify the damage to oviduct epithelium (Chapter 4).

Variations in the ability of IBV strains to cause decreases in egg production and quality was reported by Guittet et al. (1988); D274 was the least virulent while M41 and a variant strain PL84084 had the same degree of pathogenicity. Cook and Huggins

(1986) found that some variant strains of IBV caused only small decreases in egg production but had a marked effect on egg colour, unlike the more recent variant (Parsons et al., 1992) which caused a substantial decline in egg production with little loss of egg colour.

Although even the respiratory strains of IBV such as M41, show some degree of kidney pathogenicity (Jones, 1974), greater virulence for the kidney was first reported in Australia (Cumming, 1962). Since then, NIBV has been reported from USA and certain parts of Europe (Zanella, 1988; Butcher et al., 1990; Picault et al., 1991; Kinde et al., 1991; Lambrechts et al., 1993). The type of kidney lesions produced by different NIBV strains was similar but their severity varied (Albassam et al., 1986; Chandra, 1987). The Australian strain 'T' induced the most rapid and severe lesions following both i/v (Chandra, 1987) or intra-ocular (Albassam et al., 1986) inoculations of susceptible chickens. The effect of IBV on the trachea was independent of the effect on kidney (Ratanasethakul and Cumming, 1983b). A strain of NIBV, 'S' virus was less severe on the kidneys than 'T' but more severe on the tracheal mucosa. Using an i/v inoculation model to titrate kidney infectivity, Lambrechts et al. (1991) found no significant differences in the infectivity of Belgian field NIBV isolates but the infectivity of the egg-passaged virus was highly reduced.

A variant strain of IBV, strain G, was classified as being enterotropic by virtue of its greater persistence in the gut compared to the trachea (El Houadfi et al., 1986). However, no IBV strain has been demonstrated to be enteropathogenic.

Since tissue affinities are a function of the viral peplomer mediated attachment of virus to cells, changes in the spike protein may lead to altered tropism of the virus. Six of ten differences in aminoacid sequences of Gray and JMK strains of IBV were found between residues 99 and 127; hence it was postulated that this region may play a role in differences in tissue tropism exhibited by these viruses (Kwon and Jackwood, 1995). However, no correlation was found by Sapats et al. (1996) between S1 amino acid sequences and nephropathogenicity of nine Australian strains of IBV.

## 2.4. IMMUNE RESPONSES TO IBV

Immunity is considered to be either innate or acquired. Innate immunity comprises a collection of factors which resist invasion by external agents, such as physical barriers provided by skin and mucous membranes, soluble factors like lysozyme, complement and acute phase proteins and cells such as granulocytes, macrophages and natural killer

(NK) cells. The main features of innate immunity is lack of specificity and immunological memory.

Heterophils (neutrophils) constitute the 'first line of defence' against infectious agents and are the first cells to be recruited to the site of infection, following initiation of an inflammatory response. Although neutrophils are essential for host defence, they may also cause damage to host tissues (reviewed by Smith, 1994; Cassatella, 1995). In IBV-infected chickens, heterophils are the most numerous early inflammatory cells in tracheal, kidney and oviduct lesions (Crinion et al., 1971a; Purcell and McFerran, 1972; Chong and Apostolov, 1972) and in respiratory lavage fluids (Fulton et al., 1993). However, the role of these cells in IBV-infections or infact any viral infection of poultry has not been studied. Preliminary studies on the role of heterophils in IBV infections, using a heteropaenic chicken model (Kogut et al., 1993) has been performed (Chapter 10).

Macrophages act non-specifically by phagocytosing and destroying antigens by cytoplasmic enzymes or reactive oxygen intermediates. Chicken macrophages also initiate cellular and humoral responses by acting as antigen-presenting cells (Vainio et al., 1988), kill tumour cell targets via secretory factors such as tumour-necrosis factor (TNF) (Qureshi et al., 1993) and reactive nitrogen intermediates (Sung et al., 1991) and produce interleukin-1 (IL-1) (Klasing and Peng, 1987; Bombara and Taylor, 1991). The role of these cells in IBV infections is unknown.

NK cells are usually considered to be a non-B, non-T granular lymphocytes (Ortaldo et al., 1979). NK cells act in a non-specific manner killing foreign cells, virus-infected cells and tumour cells. No alterations in NK cell activity has been observed following IBV infection (Wakenell et al., 1995).

Acquired immunity results in the activation of antigen-specific effector mechanisms including B-cells (humoral), T-cells (cellular) and macrophages and the production of memory cells.

### 2.4.1. Humoral antibodies

Upon receiving proper stimuli, B-cells differentiate into plasma cells to secrete antibodies either in the presence (T-dependent antigen) or absence (T-independent antigen) of T-helper ($T_h$) cells. Chickens develop a good humoral response to IBV infections, measurable by enzyme linked immunosorbent assay (ELISA), HI or VN tests (Gough and Alexander, 1977; Wilcox et al., 1983; Monreal et al., 1985; De Witt et al., 1992). However, there is a lack of correlation between titres of circulating

antibodies and resistance to infection (Raggi and Lee, 1965; Winterfield and Fadly, 1972; Gough and Alexander, 1977). The importance of B-cells in IBV infections has been studied by depletion experiments using hormones (testosterone propionate) (Chubb, 1974), chemicals (cyclophosphamide) (Chandra, 1988; Chubb, 1974) and surgical bursectomy (Cook et al., 1991a). It was seen that in cyclophosphamide treated chickens there were increased clinical signs and more severe histopathological lesions in the kidney (Chandra, 1988) attributable to the prolonged persistence of virus in these chickens. IBV infection of a surgically bursectomised resistant line of chicken (line C) resulted in increased severity and duration of clinical infection but not mortality (Cook et al., 1991a). However, humoral antibodies seemed to protect the tracheal epithelium following secondary challenge. Presence of high titres of humoral antibodies correlate well with the absence of virus recovery from kidneys and genital tract (Gough and Alexander, 1977; MacDonald et al., 1981; Yachida et al., 1985) and protection against drop in egg production (Box et al., 1988). IBV-specific antibodies probably prevent the spread of virus from the trachea to other susceptible organs such as the kidneys and oviduct by viraemia.

The S1 glycoprotein of IBV induces VN and HI antibodies (Cavanagh et al., 1984; Mockett et al., 1984; Cavanagh et al., 1986; Niesters et al., 1987; Koch et al., 1990; Kant et al., 1992) and has been considered as the most likely inducer of protection (Cavanagh et al., 1986; Ignjatovic and Galli, 1994) but S2 and N proteins may also be important since they carried epitopes for induction of cross-reactive antibodies (Ignjatovic and Galli, 1995). The time of appearance of S1, S2 and N ELISA-antibodies have been shown to be similar, being detected two weeks after live IBV vaccination (Ignjatovic and Galli, 1995). This coincides with the appearance of VN antibodies (Gough and Alexander, 1977; Mockett and Darbyshire, 1981; Darbyshire and Peters, 1984). Epitopes on N and S2 proteins that gave rise to cross-reactive antibodies showed the same degree of conservation while S1 epitopes were shown to be marginally less conserved (Ignjatovic and Galli, 1995).

Vaccination studies with IBV have always focussed on humoral immune responses in relation to protection. Nevertheless, the lack of correlation between, antibodies and resistance, *in-vitro* strain differentiation by VN tests and *in-vivo* cross-protection results (Darbyshire, 1985) and re-excretion of virus even in the presence of high titres of circulating antibodies (Jones and Ambali, 1987) suggests that although humoral antibodies may play a role in recovery from IBV infection other immunological mechanisms may be involved.

### 2.4.2. Maternal antibodies

Maternally derived antibodies (MDA) can provide protection against IBV, but they are short-lived (Darbyshire and Peters, 1985; Cook et al., 1991b). Presence of MDA has no adverse effect on the efficacy of live IBV vaccines administered at one-day of age (Davelaar and Kouwenhoven, 1977; Davelaar and Kouwenhoven, 1981; Cook et al., 1991b). Maternally-derived IgG has also been demonstrated in tracheal washes (Mockett et al., 1987).

### 2.4.3. Local immunity

Since a primary site of IBV replication is the trachea, it has been suggested that local immunity is of fundamental importance in protection against IBV (Gomez and Raggi, 1974; Gillette, 1981; Hawkes et al., 1983). This has been exemplified by the use of an *in-vitro* challenge model using TOC from immunised chickens, for cross-protection studies (Lohr et al., 1991). IBV-specific IgA and IgG have been demonstrated in tracheal washes of infected chickens (Hawkes et al., 1983) and antibody-secreting cells were found in tracheal sections (Nakamura et al., 1991).

The Harderian gland of the chicken, located at the anterior cranial and medial aspect of the orbit, contains a large age-dependent population of plasma cells and is the source of immunoglobulins (Ig) in the lachrymal fluid (Baba et al., 1988). Davelaar and Kouwenhoven (1981) reported that the protection against IBV of day-old ocular-vaccinated chickens was localised mainly in the oculo-nasal mucosa and removal of Harderian gland caused decreased level of protection (Davelaar and Kouwenhoven, 1980). IBV-specific IgA has been demonstrated in the lachrymal fluid (Davelaar et al., 1982; Cook et al., 1992; Toro et al., 1994) and its synthesis in the Harderian gland has been shown (Davelaar et al., 1982). IgG in tears was mainly serum derived (Davelaar et al., 1982; Toro et al., 1993). IgA levels in tears appeared to be better correlated with resistance to IBV re-infection (Toro and Fernandez, 1994) rather than levels of serum antibody (Yachida et al., 1985) and was recommended for antibody profiling of chicken flocks. Cook et al. (1992) found more IBV-specific IgA in the lachrymal fluids of chicken lines resistant to IBV while antibody in tracheal washes were similar.

Although IBV has been shown to multiply in the gut (Lucio and Fabricant, 1990; Ambali and Jones, 1990), Lutticken et al. (1988) could not detect any antibodies in gut fluid. A study on the local antibody production in IBV-infected hens has been done in Chapter 6.

### 2.4.4. Cellular immunity

Reports concerning a role for cell-mediated immunity in protection against IBV are limited. Antigen-specific proliferation of T-lymphocytes in IBV-infected or vaccinated chickens has been demonstrated (Timms et al., 1980; Timms and Bracewell, 1981; Timms and Bracewell, 1983). In some chickens, a positive correlation between lymphoproliferative responses and resistance to challenge has been shown (Timms and Bracewell, 1981). A T-cell epitope has been identified in the IBV 'N' protein (Boots et al., 1991) and has been shown to induce anti-viral responses (Boots et al., 1992).

Mouse monoclonal antibodies (Mab) that distinguish between different chicken T-lymphocytes have been described (Lillehoj et al., 1988; Chan et al., 1988). The CD4 and CD8 antigens are found on two main populations of T-cells, T-helper ($T_h$) and T-cytotoxic/supressor ($T_{c/s}$) cells respectively. In mice two sub-populations of $T_h$ cells have been defined based on their cytokine secretion profiles (Mosmann et al., 1986). $T_h1$ cells secrete IFN and also tend to secrete IL-2, while $T_h2$ cells secrete IL-4 and also IL-5, IL-6 and IL-10. The cell type whose secretions predominate may help to determine the outcome of infections (Sher and Coffman, 1992).

CD4 and CD8 cells have been shown in sections of trachea, lung and kidneys of chickens infected with NIBV (Janse et al., 1994). No studies have been reported on the kinetics of T-cell subset changes following IBV infections using flowcytometry. Comparison of T-lymphocyte subset changes between a resistant and sensitive inbred line of chicken (Bumstead et al., 1989) would give more information on the role of T-cells in immunity to IBV. However, the general importance of T-cell immunity in infectious diseases has been studied by depletion experiments using T-cell suppressor drug, CSP (Fitzgerald et al., 1995; Suresh and Sharma, 1995; Lillehoj, 1987) or by adoptive transfer studies among inbred lines of chickens (Fahey et al., 1984). The role of T-cells in IBV infections was elucidated using CSP to supress T-cells (Chapter 9).

Chubb et al. (1987) demonstrated the presence of cytotoxic lymphocytes (CTL) in the spleen and peripheral blood following IBV infection using adherent cells as target cells and neutral red as indicator of lysis. However, Wakenell et al. (1995) could not demonstrate CTL using kidney cells as target and the conventional chromium-release assay.

Delayed type hypersensitivity (DTH) responses were induced in response to live IBV (Chubb et al., 1988) and to affinity-purified S1, S2, N and M proteins (Igjnatovic and Galli, 1995). However, cross-reactivity of cellular responses to serologically different IBV strains has not been studied. This has been done in Chapter 11.

Cytokines secreted in response to a general stimulus such as concanavalin A (Con A) or by specific antigen are important mediators of cellular immunity. T-cell growth factor (TCGF) or interleukin-2 (IL-2) and interferon γ (IFN-γ) are among the most important soluble factors produced by lymphocytes. IL-2 interacts with cells of the immune system to influence their differentiation and effector functions, including secretion of other lymphokines. IFN-γ regulates several immunological reactions such as the activation of macrophages and enhancement of immunogenicity through expression of major histocompatibility complex antigens. Only IFN-γ has been studied in relation to IBV but results are conflicting. Otsuki et al. (1988) detected variable levels of IFN-γ in chickens with various strains of IBV whereas other workers (Lomniczi, 1974; Holmes and Darbyshire, 1978) could not detect IFN-γ in serum or organ cultures of chickens infected with IBV. Furthermore, whether IBV is susceptible to anti-viral effects of IFN-γ is controversial (Holmes and Darbyshire, 1978; Otsuki et al., 1979b). Otsuki et al. (1991) found no differences in IFN-γ levels of IBV-resistant and IBV-sensitive lines of chickens.

## 2.5. DIAGNOSIS

The clinical signs for IBV are not pathognomonic; hence diagnosis on this basis alone is not possible. However, severity of clinical signs under experimental conditions is used for assessing protection afforded by vaccines following challenge (Davelaar and Kouwenhoven, 1977; Davelaar and Kouwenhoven, 1980; Yachida et al., 1985). Diagnosis of IBV is based on isolation and identification of the virus and rising IBV antibody titres.

### 2.5.1. Isolation and identification of the virus

The trachea being a primary site of virus replication, is the preferred sampling site early in the infection. Cloacal swabs, faeces, caecal tonsils, kidney, oviduct, lungs or airsacs may be preferred later in the course of infection (Cook, 1968; Davelaar et al., 1984). Rarely, these samples may be inoculated in to susceptible chickens which produce characteristic respiratory signs in 18 - 36 hrs, if IBV is present. Virus isolation is routinely performed by inoculation into embryonated chicken eggs (ECE) or TOC. Several passages may be required in ECE before typical changes are seen. These include congestion and increased prominence of blood vessels in chorio-allantoic membrane, embryo stunting and persistence of mesonephros containing urates (Lukert, 1965; Butler et al., 1972b). TOC is preferred for primary isolation as no adaptation of the virus is required for growth and induction of ciliostasis (Cook et al., 1976). However, IBV must be adapted to grow in chicken embryo kidney cell cultures

(Gillette, 1973). Hence cell culture systems are not used for primay isolation of the virus.

More rapid identification of the virus is possible by techniques such as IF of tracheal smears or sections (Yagyu and Ohta, 1990), IP of tissue sections (Naqi, 1990; Owen et al., 1991), IF on allantoic cells (Clarke et al., 1972; Endomunoz and Faragher, 1989), IP on infected chorio-allantoic membranes (Naqi, 1990), IF on unfixed infected TOC rings (Bhattacharjee et al., 1994), antigen-capture ELISA (Hesselink et al., 1988), IF or IP on cell cultures (Toro et al., 1987; Csermelyi et al., 1988) and electron microscopy of concentrated allantoic fluids (McFerran et al., 1971). Other less-common methods of identification, used presently, are agar gel immunodiffusion (AGID) using tracheal mucus or infected chorio-allantoic membranes (Witter, 1962) or tests based on the ability of IBV to interfere with the replication of Newcastle disease virus (NDV) (Raggi and Pignattelli, 1975; Cubillos et al., 1991). Recently polymerase chain reaction (PCR)-based (Andreasen et al., 1991) methods have been used to identify IBV in tracheal swabs (Kwon et al., 1993a) and allantoic fluids (Jackwood et al., 1992; Zwaagstra et al., 1992). The PCR technique identifies only the viral RNA and not the infectious virus.

Isolation and identification of IBV should be followed up with strain differentiation as this is an essential prerequisite for constant antigenic monitoring of IBV isolates. This is normally done by the VN test using primary antisera against the strains prevalent in that region. VN tests have been performed in ECE (Davelaar et al., 1984), TOC (Johnson and Marquardt, 1975; Darbyshire et al., 1979; Cook, 1984; Cook and Huggins, 1986) or in cell cultures using plaque reduction (Hopkins, 1974), neutralisation of immunofluorescent foci (Csermelyi et al., 1988) or IP (Toro et al., 1987) as indicator systems. Although HI tests have also been used for typing IBV isolates (King and Hopkins, 1984; Lashgari and Newman, 1984; Brown and Bracewell, 1985), the test of choice is the VN test (Cook et al., 1996) because of extensive cross-reactivity and difficulty in interpretation of HI results (Cook et al., 1987). Both the VN and HI tests employ primary antisera since sera produced by repeated inoculations of the virus have been shown to be cross-reactive (Gelb and Killian, 1987; Brown and Bracewell, 1988).

The availability of serotype-specific Mab (Mockett et al., 1984; Niesters et al., 1987; Wainwright et al., 1989; Ignjatovic and McWaters, 1991; Karaca et al., 1992) has enabled serotyping of IBV using antigen-capture ELISA (Cavanagh et al., 1992a; Naqi et al., 1993) or IF directly on sections of infected tracheas obtained from field cases (De Wit et al., 1995). Other reported methods for comparison of IBV strains include oligonucleotide fingerprinting (Clewley et al., 1981; Kusters et al., 1987; Karaca et al.,

1990), limited proteolysis of viral glycoproteins (Cavanagh and Davis, 1987), estimation of iso-electric points (Sadasiv et al., 1991), dot-blot hybridisation using digoxigenin-labelled cDNA probes (Nagano et al., 1993), PCR followed by restriction fragment length polymorphism (RFLP) studies (Lin et al., 1991a; Lin et al., 1991b; Kwon et al., 1993b; Avellanda et al., 1994), strain-specific nested PCR (Cavanagh et al., 1995) and sequencing (Cavanagh et al., 1995; Sapats et al., 1996). However, Jia et al. (1995) cautioned on the use of these recent typing methods based on dot-blot hybridisation, PCR or RFLP, since every field isolate of IBV may have an unique genetic nature as a result of recombination in more than one gene.

### 2.5.2. Demonstration of antibody

The most commonly used serological procedures to demonstrate rising IBV-antibody titres include agar gel precipitation (AGP), IF, ELISA, HI and VN tests. AGP, IF and ELISA detects only group-specific antigens and cannot be used to differentiate between strains (Lucio and Hitchner, 1970; Marquardt et al., 1981; Mockett and Darbyshire, 1981; Wilcox et al., 1983; Zellen and Thorson, 1987). The VN test is preferred to HI for serotyping IBV (Cook et al., 1987; Cook et al., 1996). However, HI is valuable for monitoring serological responses to IBV vaccination (King and Hopkins, 1983) and diagnosing field IBV infections (Macpherson and Feest, 1978). Karaca and Naqi (1993) developed a blocking ELISA for detection of serotype-specific antibodies, which combined the sensitivity of indirect ELISA and the serotype-specificity of the VN test.

An ELISA to detect IgM has been shown to indicate recent IBV infection (Gillette, 1974; Mockett and Cook, 1986; Martins et al., 1991; Bhattacharjee et al., 1995). However, IgM antibodies have to be separated either by sucrose density gradient centrifugation (Gillette, 1974) or column chromatography (Mockett and Cook, 1986) before performing an ELISA. The availability of an antibody-capture ELISA for IgM assay would facilitate its applicability to field cases.

## 2.6. CONTROL

There is no specific treatment for IB. Proper management practices will help to reduce losses due to IBV infections. Antibiotics may reduce secondary bacterial infections following IBV infections. Supplementation of 300 - 330 ppm of ascorbic-acid in the feed has been shown to reduce the severity of tracheal lesions following IBV challenge (Davelaar and Van den bos; 1992). The use of electrolyte replacers in drinking water has been recommended to reduce losses from nephritis (Cumming, 1969); however, the rationale has been questioned by Condron and Marshall (1985). They suggested that

since sodium loss in NIBV infections is due to reduction in absorbtion, sodium supplementation may aggravate water losses.

The most common method of control is by the use of live attenuated or killed vaccines against IBV. Vaccine strains are selected to represent the antigenic spectrum of isolates in a particular region. Administration methods include eyedrop, intra-nasal, intratracheal, coarse spray, aerosol or drinking water (Andrade et al., 1983; Ratanasethakul and Cumming, 1983a). Embryonal vaccination has also been sucessfully used experimentally (Wakenell and Sharma, 1986; Wakenell et al., 1995).

IBV strains used for live vaccines are attenuated by serial passages in ECE (Klieve and Cumming, 1988) or in cell culture (Wakenell et al., 1995). Attenuation by incubation of IBV-inoculated eggs at a lower temperature ($28^0$C or $31^0$C) has been shown to be quicker (Klieve and Cumming, 1988; Gelb et al., 1991). The level of attenuation is assessed by inoculation of susceptible chicks and monitoring clinical symptoms or severity of lesions. Cook et al. (1989) have shown that inoculation of poults with attenuated strains of TRTV produced less damage to trachea as assessed by tracheal ciliary activity in organ cultures. However, there is no *in-vitro* method for assessment of attenuation of virus strains. A model based on differences between virus titres determined by ciliostasis, IF and organ culture infectivity has been proposed for use as an *in-vitro* attenuation marker for IBV vaccines (Chapter 5).

### 2.6.1. Methods of assessing protection

Recently, two criteria for assessment of protection to IBV have been used : assessment of protection at the level of trachea and protection against mortality, following challenge. Since the respiratory tract is the primary target organ of IBV infections, resistance at that site would prevent the challenge virus establishing itself in other organs by viraemia (Andrade et al., 1982). Protection at tracheal level, following challenge, has been assessed by absence of respiratory signs (Raggi and Gomez, 1975), failure to recover challenge virus from the trachea (Hitchner et al., 1964; Winterfield et al., 1972; Hofstad, 1981; Gelb et al., 1981) and absence of histopathological lesions in the trachea (Davelaar and Kouwenhoven, 1977; Davelaar and Kouwenhoven, 1980). Darbyshire (1980) first reported the approach of observing the presence or absence of ciliary activity of tracheal explants prepared from vaccinated chickens after challenge, as a measure of protection. Complete agreement was reported between results of ciliary activity, virus isolation and histopathological changes used as a measure of protection (Andrade et al., 1982; Marquardt et al., 1982; Snyder et al., 1983; Andrade et al., 1983; Klieve and Cumming, 1990). However, Yachida et al. (1985) reported that observation of clinical signs together with tracheal ciliary activity is

needed to determine the immune status of chickens vaccinated a long period before, although respiratory signs was found not to be an exact measurement of protection (Winterfield et al., 1972; Darbyshire, 1980). No correlation was observed between serum antibody titres and protection of trachea based on ciliostasis, respiratory signs and tracheal virus isolations (Raggi and Lee, 1965; Gough and Alexander, 1977; Darbyshire and Peters, 1984; Yachida et al., 1985). Evaluation of tracheal ciliary activity is routinely used to assess protection afforded by existing vaccines to variant strains of IBV (Cubillos et al., 1991; Parsons et al., 1992).

Although trachea is the primary target organ of IBV and is the portal of entry of the virus, IBV can also cause systemic infection in other tissues especially kidneys and oviduct. Darbyshire and Peters (1984) have suggested that immunity of the tracheal mucosa does not necessarily indicate a more generalised protection. The development of an experimental model for IBV using combined inoculations of a pool of strains of IBV and *E.coli* (Smith et al., 1985) which resembled the natural disease enabled its use for the study of protection, based on the fate of the intact animal following challenge (Cook et al., 1986; Cubillos et al., 1991; Cook et al., 1991b). Cubillos et al. (1991) reported that a field isolate of IBV in Chile together with *E.coli*, caused mortality or peritonitis in 50% of inoculated chicks, but caused least damage to the tracheal epithelium probably due to the variable tropism of that virus. It would be interesting to compare the degree of protection afforded by IBV using these two criteria.

Other methods used to assess protection are quantitative measurement of virus titres following challenge of vaccinated chickens (Darbyshire, 1985), vaccinating dose required to prevent multiplication of challenge virus in the lungs (Arvidson et al., 1990), protection of ciliary activity of tracheal explants from immunised chickens following *in-vitro* challenge (Lohr et al., 1991) and by placing IBV-vaccinated sentinel chickens with commercial broiler chickens and determining the incidence of IBV infections after a week of field exposure (Gelb et al., 1989).

Protection at the level of kidneys has been assessed by reduction in mortality following challenge (Chubb, 1973; Meulmans et al., 1987; Lambrechts et al., 1993; Pansaert and Lambrechts, 1994), incidence of kidney IF (Lambrechts et al., 1993) and histopathological changes in the kidneys (Albassam et al., 1986). Protection at the level of oviducts has been assessed by the ability of vaccines to prevent loss in egg production induced by the challenge virus (Box et al., 1980; Box and Ellis, 1985; Box et al., 1988). No method is available to assess protection on the basis of prevention of direct damage to the oviducts. This lacuna has been addressed in Chapter 7.

# CHAPTER 3

## GENERAL MATERIALS AND METHODS

**CONTENTS** **PAGE NUMBER**

# CHAPTER 3

## GENERAL MATERIALS AND METHODS

This chapter describes the general materials and methods used routinely throughout the experimental work. Details of specialised procedures used in specific experiments are included in relevant chapters. The composition of media, reagents and buffers is presented in the Appendix.

### 3.1. EMBRYONATED CHICKEN EGGS

ECE were used for virus propagation, preparation of TOC, preparation of chicken embryo fibroblast (CEF) cultures and as a source of day-old specific-pathogen-free (SPF) chicks.

#### 3.1.1. Source of the eggs

The SPF eggs were obtained from a commercial supplier (Wickham laboratories, Wickham, Hants, U.K.). The parent flock was free of major infectious disease agents including IBV and chicken anaemia virus.

#### 3.1.2. Incubation

All eggs were washed with the egg shell disinfectant, Rychor (Rychor Ltd., Oakenshaw Chemical Works, Clayton-le-Moors, Accrington, England) by an electrically-operated egg washing machine. The eggs were allowed to dry at room temperature and usually set for incubation within the first week of being laid. Incubation of eggs was carried out in a commercial incubator with automatic hourly turning. Fertile eggs were used at the age required.

#### 3.1.3. Egg inoculations

Fertile eggs of 9 - 10 days of incubation were used for the propagation of IBV. The eggs were marked around the air space and also at a point on the periphery of the air-space where no major blood vessels could be seen on the chorioallantoic membrane. This point was punctured with a pin disinfected with 70% alcohol and the inoculum was introduced in to the allantoic cavity by a syringe equipped with a 26 gauge needle. The inoculation site was sealed with molten paraffin wax and eggs were incubated at $37^0$C in a still incubator and examined daily.

## 3.2. ORGAN CULTURES

### 3.2.1. Tracheal organ cultures

The method for the preparation of TOC has been described by Cook et al. (1976). Briefly, the embryos were removed from fertile 19- or 20-day old SPF eggs and the tracheas collected from them. They were trimmed of excess fat and other tissues and placed in a petri dish containing warm ($37^0$C) TOC medium (see Appendix). They were then cut into 0.6mm thick rings using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, UK) and each ring was placed in a sterile tube (Nunc, Life Technologies, Paisley, Scotland) containing 0.7ml of medium. Tubes were rotated in a roller drum at 8 revolutions per hour at $37^0$C. After 24 hours, the rings were checked for ciliary activity and used as required.

### 3.2.2. Induction of precocious oviducts and OOC

Precocious development of oviducts was induced by oestrogen treatment of unsexed chicks following the method of Pradhan et al. (1984) with modifications. SPF chicks were inoculated with 1mg (0.2 ml) of oestradiol-benzoate (Intervet UK, Cambridge, UK) subcutaneously on three occasions with three days interval between injections. Two or three days after the final injection, oviducts were collected from female chicks at post-mortem.

From these precociously-induced oviducts, OOC were prepared similar to the method described above for TOC except that the medium used was M199 (see Appendix).

## 3.3. EXPERIMENTAL CHICKENS

Embryonated eggs were checked for their fertility on the 18th day of incubation and were transferred into a tray kept at the bottom of the incubator in a static condition for hatching at 21 days.

### 3.3.1. Housing and management

The chicks were maintained in isolation pens or flexible isolators equipped with a supply of filtered air under negative pressure. Foot baths with iodophor disinfectant (FAM, Evans Vanodine International PLC, Lancashire, UK) and protective clothing including overalls, yashmasks, latex gloves and boots were used at all times when visiting birds. Heat was supplied from 1.5 KW radiant electric heaters. The chickens were placed on wood shaving litter in the isolation pens or on wire cages in isolators. Feed and water were provided *ad-libitum*.

## 3.4. VIROLOGICAL METHODS

### 3.4.1. IBV Strains

Several field and vaccine strains of IBV belonging to different serotypes were used in this study. Their details are given in respective chapters.

### 3.4.2. Processing of samples for virus isolation

In various experiments virus isolation was attempted either from swabs or macerated tissues. After sampling, the swabs were dipped in to 1 ml of TOC medium containing x10 the amount of antibiotics, shaken vigorously and stored at -70$^0$C until required. Tissues were macerated by grinding in a sterile pestle and mortar using sterile sand and a small volume of medium. Then more medium was added to the ground tissue to make a final 1 : 10 (w/v) dilution of the sample. Following centrifugation at 1500 g for 10 min, the supernatants were collected and stored at -70$^0$C until used.

### 3.4.3. Virus isolation in TOC

Twenty four hours after preparation, each tracheal ring was checked microscopically and only those showing vigorous ciliary beating were used. The medium was removed and rings were each inoculated with 0.1 ml of the sample suspension. Virus was allowed to adsorb at 37$^0$C for one hour. After adsorption, the inoculum was removed, the rings were washed with medium and then overlaid with 0.7 ml of TOC medium and maintained in a roller drum at 37$^0$C. The tubes were examined daily and the occurrence of ciliostasis (cessation of ciliary activity accompanied by loss of ciliated epithelium) was taken to be indicative of presence of virus. All the samples were passaged up to three times at 4-day intervals before being considered negative.

### 3.4.4. Virus titration

Replicates of 3 - 5 tubes of TOC or OOC were inoculated with ten-fold dilutions of samples as described above. The occurence of complete ciliostasis was taken as the endpoint. The titres were calculated according to the method of Reed and Muench (1938) and expressed as median ciliostatic doses ($\log_{10}$ $CD_{50}$) per ml.

### 3.4.5. Identification of virus

To demonstrate virus-specific antigens on unfixed TOC rings or cryostat sections of tissue, an indirect IF technique was used either with hyperimmune sera (see below) or Mab to IBV (see Chapter 9).

Identification of ciliostatic virus in unfixed TOC rings was performed following the method described by Bhattacharjee et al. (1994). Briefly, 24 hrs after inoculation, TOC

rings were placed in a 48-well cell culture plate (Costar UK Ltd., High Wycombe, Bucks) and washed with phosphate buffered saline (PBS) pH 7.2 using a plate shaker (Titertek, Irvine, Scotland). Then, each well received 0.15 ml of optimally diluted antiserum against IBV strain G and the plate was incubated for one hour at $37^0$C. After washing, the cultures were incubated with 0.15 ml of optimally diluted commercial affinity-purified goat anti-chicken IgG (Sigma, Poole, Dorset) for another hour. Following washing, the rings were mounted on teflon-coated slides (ICN Biomedicals Inc., Costa Mesa, California, USA) with fluoromount-G (Southern Biotechnology Ltd., Birmingham, USA ) and examined under a fluorescent microscope (Leica UK Ltd., Milton Keynes, UK) equipped with incident UV/blue illumination. Specific fluoresence in the epithelial cells of TOC was taken as an indicator of the presence of IBV-specific antigens.

Small pieces of tissues were collected in aluminium foil cups containing an embedding medium, OCT compound (Tissue Tek, Miles Inc., Elkhart, Indiana, USA) and snap-frozen in liquid nitrogen. Each sample was then collected in a self-sealing polythene bag and stored at $-70^0$C until used for sectioning. Cryostat sections were cut at a thickness of 4 - 6 μ, collected onto poly-L-lysine coated slides (Sigma), air-dried and fixed in absolute acetone at room temperature for 10 minutes.

The acetone-fixed sections were rinsed briefly in PBS and then flooded with a pre-determined optimal dilution of hyperimmune serum or Mab and incubated at $37^0$C for one hour in a humid sandwich box. Excess primary antibody was drained off and the slides washed in two changes of PBS for 15 minutes each in a bath using a magnetic stirrer. After washing, the appropriate secondary antibody was added and incubated similarly. Following washing, the slides were mounted and examined as described above.

Sections of tissues from uninfected chickens and the use of sera prepared from control birds in place of primary antibody, were used as controls to assess the specificity of IF staining.

### 3.5 PURIFICATION OF VIRUS

IBV was propagated by passage through the allantoic cavity of 9- to 10-day old ECE as described above. After 48 hours, the live embryos were chilled and their allantoic fluids were harvested and clarified. Supernatants were collected and centrifuged at 20,000 g for 90 minutes in a swing-out rotor (AH 629) using an ultracentrifuge (Sorvall Instruments, Dupoint, USA). The pellet was resuspended in a small volume of PBS

and layered on a continous sucrose gradient (see Appendix) and centrifuged at 50,000 g overnight at $4^0$C. Fractions were collected in volumes of 1.5ml from the bottom of the tube and each fraction was coated onto ELISA plates and checked for the presence of antigen using known hyperimmune chicken serum. The density of each fraction was determined by weighing 100 µl volumes. Fractions giving the highest optical density (OD) in ELISA and within the density range of 1.16 to 1.19 were pooled, diluted in PBS and centrifuged at 75,000 rpm at $4^0$C for 4 hours. The pellet was resuspended in a small volume of PBS and this constituted the purified viral preparation. The optimum dilution of this virus for use in ELISA was determined by checker-board titrations. The immunoglobulin class-specific ELISA procedure is described in Chapter 6.

## 3.6. SEROLOGY

### 3.6.1. Preparation of hyperimmune serum

Sixteen week-old male chickens were infected oculo-nasally with 4.5 $\log_{10}$ $CD_{50}$ of IBV strain G . Three weeks later, a similar booster dose was given and the birds were bled after two-weeks. Sera separated from these birds were used for IF, VN and HI tests.

Day-old SPF chickens infected with IBV strain M41 were bled 4 weeks later and the sera used in HI or VN tests.

### 3.6.2. Haemagglutination-inhibition test

The HI test was used to either to assess antibody levels in samples (Chapter 6) or for IBV strain differentiation (Chapter 8). IBV strains were propagated in ECE as described in Section 3.1.3. The allantoic fluids were collected 30 - 48 hours later and clarified at 1500 rpm for 10 minutes in a laboratory bench centrifuge. Then the virus was pelleted at 30,000 g for 40 minutes in an ultracentrifuge and resuspended at a 125-fold concentration in PBS. The enzyme phospholipase-C type 1 (Sigma) at a concentraton of 5 units per ml was added to the concentrated virus to give a final dilution of 1 unit per ml in a 100-fold concentration of the virus. The mixture was incubated at $37^0$C in a water bath for 2 hours after which it was assayed for its haemagglutination (HA) activity.

The HI procedure was performed using 4 HA units of the treated virus (final concentration) and 0.5% of washed chicken red blood cells (RBC). Briefly, two-fold serial dilutions of samples were made in a volume of 25 µl, followed by the addition of virus containing 8 HA units in 25 µl. After incubation at room temperature for 30

minutes, 50 µl of 0.5% chicken RBC suspension was added and the test read after incubation at $4^0$C for 45 minutes. The HI titre was taken as the highest dilution of the sample at which HA was inhibited. The dilution of virus added was counter-checked by back titrations and the specificity of HI was assessed by using samples collected from age-matched control chickens.

### 3.6.3. Serum neutralisation test (ß-method)

Serial two-fold dilutions of heat-inactivated ($56^0$C for 30 minutes) serum were made in TOC medium and each dilution was mixed with an equal volume of virus suspension containing 100 $CD_{50}$ in 0.1 ml. The mixture was incubated at room temperature for 30 minutes. After removing the medium from TOC tubes, 0.2 ml of the virus-serum mixture was added into each tube and incubated at $37^0$C for one hour. Then, the tubes were overlaid with 0.5 ml of TOC medium and incubated in a roller drum at $37^0$C. The test was read after 72 - 96 hours by examining the cultures for ciliostasis.

To cross-check the virus dose, a back titration of the diluted virus was performed. A series of half-log dilutions of the virus used for the assay were made by dispensing 1.1 ml of TOC medium into 6 bijou bottles. To the first bottle was added 0.5 ml of the virus used for the test. The mixture was shaken and 0.5 ml of the solution was transferred in to the second bottle and so on, giving a series of $\log_{10}$ 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 dilutions.

The endpoint titres for both test sera and the back titration were calculated according to the method of Reed and Muench (1938). The test was considered valid if the endpoint of virus back-titration was $\log_{10}$ 2.0 ± 0.5.

# CHAPTER 4

## AN *IN-VITRO* COMPARISON OF THE VIRULENCE OF SEVEN STRAINS OF INFECTIOUS BRONCHITIS VIRUS USING TRACHEAL AND OVIDUCT ORGAN CULTURES.

**CONTENTS** **PAGE NUMBER**

# CHAPTER 4

## AN *IN-VITRO* COMPARISON OF THE VIRULENCE OF SEVEN STRAINS OF INFECTIOUS BRONCHITIS VIRUS USING TRACHEAL AND OVIDUCT ORGAN CULTURES.

### 4.1. INTRODUCTION

Studies involving the virulence and tropism of different strains of IBV for the reproductive tract of chickens have been scarce (McMartin, 1993). However, evidence for differences between strains in virulence for the oviduct were reported by Crinion and Hofstad (1972a): Massachusetts and Australian T strains were virulent while Connecticut and Iowa 609 were not. Repeated embryo passage of Massachusetts virus reduced its virulence for the oviduct (Crinion and Hofstad, 1972b).

TOC have been extensively used for the cultivation and assay of IBV (Cook et al., 1976) and for taxonomic (Darbyshire et al., 1979) and cross-protection (Darbyshire, 1980) studies. Pradhan et al. (1984) used an OOC system for studying the pathogenicity of a field isolate of IBV, belonging to the Massachusetts serotype, for the chicken oviduct but they did not compare the pathogenicity of different strains.

For TOC, occurrence of ciliostasis is routinely used to assess infectivity of IBV. It was not known whether the same criterion would be useful for OOC. Since epithelial cells are the target cells for IBV in the oviduct (Jones and Jordan, 1971), a calmodulin (CAM) assay was also used in an attempt to quantify oviduct epithelial cell damage caused by IBV. Calcium is recognised as a mediator of many cell processes and a calcium-modulating protein, CAM acts as an intracellular regulator of calcium ions. The role CAM plays in ciliary activity of epithelial cells makes this molecule a useful measure of cell health and membrane permeability (Cheung, 1982; Verdugo et al., 1983). This assay has been used previously for studying pathogenicity of mycoplasma and ureaplasma strains for equine and bovine oviductal explants (Bermudez et al., 1992; Smits et al., 1994).

Thus, the aim of the present study was three-fold: (i) to assess the pathogenicity and tropism of different IBV isolates for trachea and oviduct using TOC and OOC (ii) to compare the virulence of IBV isolates for oviducts and (iii) to adapt a quantitative assay for CAM to estimate the epithelial cell damage to chicken oviducts caused by IBV.

## 4.2. MATERIALS AND METHODS

### 4.2.1. Viruses

Seven strains of IBV were used in this study and their details are shown in Table 4.1. Strains 6, 7, 8 and 25 were isolated in U.K. from gut tissue or cloacal swabs and the year of isolation is indicated by the second figure in the table. Strain 371/85 belongs to a new unnamed serotype. All these strains had undergone less than 10 passages in TOC. Strains G and M41 have been maintained in this laboratory for several years and have had numerous passages in TOC. The 793/B-like variant was isolated from the field (Chapter 8) and is antigenically identical to strain 793/B described by Gough et al (1992). All the viruses except M41, G and 793/B-like were kindly supplied by Dr.Cook (Intervet Laboratories, Houghton, U.K.). All were passaged once in TOC and once in 10-day old embryonated SPF chicken eggs. The allantoic fluids were harvested 48 hours post-inoculation (p.i.). The viruses were titrated in TOC using complete ciliostasis as the end-point (Cook et al., 1976). Titres were calculated using the method of Reed and Muench (1938).

**Table 4.1. IBV strains used in this study.**

| Strain | Designation | Serotype | Reference |
|---|---|---|---|
| 6 | 1432/81 | D207 | Cook (1984) |
| 7 | 165/82 | D3896 | Cook (1984) |
| 8 | 123/82 | D3896 | Cook (1984) |
| 25 | 371/85 | variant | Cook (unpublished) |
| G | | enterotropic variant | El Houadfi et al. (1986) |
| M41 | | Massachusetts | Ambali and Jones (1991a) |
| 793/B-like | | variant | Chapter 8 |

### 4.2.2. Serum

Hyperimmune serum was raised against IBV strain G in adult SPF cockerels as described in Chapter 3 and used in indirect IF tests for detection of group-specific antigen of all the strains tested.

### 4.2.3. Induction of precocious oviducts in chicks

Precocious development of oviducts was induced by oestrogen treatment of unsexed chicks following the method detailed in Chapter 3. Briefly, SPF day-old chicks were inoculated with 1mg of oestradiol benzoate (Intervet, U.K.) subcutaneously on days 1,

5 and 9 post-hatch. Two or three days after the last injection, chicks were killed and oviducts were collected from females. Males were discarded at post-mortem.

### 4.2.4. Oviduct organ culture

OOC were prepared as described in Chapter 3. After 24 hours of culture, the rings were checked for ciliary beating and used as required.

For virus titrations, IF and CAM assays, 1mm transverse slices of oviduct tissue were used. After infection, cultures were left stationary for one hour at $37^0$C. After adsorption, the inoculum was removed and fresh medium added after washing the tissue.

### 4.2.5. Tracheal organ culture

For virus titrations, TOC were prepared using 19- or 20-day old embryonated SPF chicken eggs (Chapter 3). For ciliary scoring, TOC were prepared using tracheas collected from the same female chicks that provided the oviducts.

### 4.2.6. Measurement of ciliary activity

The proportion of the periphery of tracheal and oviduct rings with ciliary beating was estimated on a daily basis and expressed as percent peripheral ciliary activity (PPCA). The ciliary vigour was scored as follows: 3 for fast harmonic beating, 2 for moderate coordinated beating, 1 for slow incoordinated beating and 0 for lack of beating (Bermudez et al.,1990). The relative ciliary activity (RCA) was calculated by multiplying the PPCA by the score for ciliary vigour as described by Stadtlander et al. (1991). The time taken, in days, for a 50% reduction in RCA was determined from the graphs. For seven IBV strains, two input doses of 2.0 and 4.0 $\log_{10}$ $CD_{50}$ were used on both tracheal and oviduct rings. All the TOC used in this study had an RCA of 100% before infection. The OOC had an RCA of between 80-100% on day 0, which was converted to 100% and the corresponding decline evaluated.

### 4.2.7. Immunofluorescence of OOC

Oviduct tissues were infected with 4.0 $\log_{10}$ $CD_{50}$ of each strain. The infected tissues were collected on days 1-5 p.i., frozen in an embedding compound (OCT, Tissuetek) using liquid nitrogen and stored at $-70^0$C until tested. Five $\mu$m thick sections were cut using a cryostat and IF staining done as described in Chapter 3. The fluorescence of epithelial cells was scored on a 0-3 scale as follows: 0 for no fluorescence, 1 for a few fluorescing cells, 2 for discrete areas of fluorescence and 3 for extensive fluorescence. Controls included IF-staining of uninfected cultures and also of infected tissues but

with normal chicken serum. Three trials were done and two sections were examined per trial. Thus the scores for each isolate per day was a mean of six individual scores.

### 4.2.8. Virus titres in OOC

Oviduct tissues were infected with 2.0, 4.0 or 6.0 $\log_{10}$ $CD_{50}$ of each IBV strain and supernatants were collected between days 1-5 p.i. Two trials were done, each using five tubes per input dose. The pooled harvests of 10 tubes were titrated in TOC and the titres calculated according to Reed and Muench (1938).

### 4.2.9. Calmodulin assay

The CAM assay was done on individual supernatants of infected and control oviduct tissues as collected for virus titration, but with the 4.0 $\log_{10}$ $CD_{50}$ input dose only. The method used was that of Bermudez et al (1992) with slight modifications. All the reagents for the assay were purchased from Sigma (Poole, Dorset, UK) except standard CAM which was from Boehringer Mannheim UK Ltd. (Sussex, England). A phosphate liberation end-point assay, originally described by Sharma and Wang (1986) was used. In this assay the CAM content is estimated by the degree of activation of CAM-deficient, CAM-dependent phosphodiesterase (obtained from bovine heart). The adenosine monophosphate generated from adenosine 3' 5' cyclic monophosphate is hydrolysed by 5' nucleotidase (obtained from *Crotalus* venom) to generate inorganic phosphate.

The reaction mixture consisted of 175$\mu$l assay buffer (100mM Tris, 40mM imidazole, 5mM magnesium, 0.5mM $CaCl_2$, pH 7.5), 25$\mu$l of 15mM cAMP, 25$\mu$l of 4U/ml 5' nucleotidase, 25$\mu$l of 0.15U/ml phosphodiesterase and between 5 to 80 $\mu$l of purified CAM (1ng/$\mu$l) or 100 $\mu$l of heat treated samples. Reactions were initiated by the addition of cAMP after the other ingredients were pre-incubated at $37^0$C for 5 minutes. Reactions were terminated after 30 minutes at $37^0$C by adding 250$\mu$l of 10% SDS buffered to pH 7.0 with 20 mM Tris. Phosphate was detected by the addition of 500$\mu$l of a chromogenic solution containing freshly prepared 5% (v/v) ferrous sulfate and 10% (v/v) of a stock solution of 10% ammonium molybdate in 10N $H_2SO_4$. Absorbance was determined at 720 nm in a spectrophotometer in batches of 10 tubes using medium as blank.

A standard curve was generated by plotting absorbance versus amount of purified CAM. The CAM concentration of media samples were calculated from the standard curve. The specificity of the assay was established using a CAM inhibitor R2457 (calimidazolium) at 10$\mu$M concentration which reduced the absorbance by 95%. All the samples were processed as described by Bermudez et al. (1992) prior to the assay.

### 4.2.10. *In-vitro* sensitivity of TOC and OOC

For each virus, three sets of cultures were inoculated: (i) tubes containing one slice of OOC and one of TOC, (ii) tubes containing only TOC and (iii) tubes containing only OOC. The presence of fluorescing cells in (ii) or (iii) indicated that these tissues were innately susceptible to the inoculated strain. Specific fluorescence at one day p.i. in only one tissue in (i) indicated that that tissue was more susceptible that the other, while positive staining in both at this time indicated equal susceptibility. Five strains (6, 25, G, M41 and 793/B-like) were used at an input dose of 4.0 $\log_{10}$ $CD_{50}$ and three replicate trials were done.

## 4.3. RESULTS

### 4.3.1. Precocious oviducts

Following oestrogen inoculations, the chicks remained apparently normal and active. At autopsy, the mean length of the oviducts when extended, was 6.17cm (n=23) and their mean weight was 1.03 g (n=23). Up to 40 OOC rings could be obtained from each oviduct. Figure 4.1 shows precociously-induced oviducts in an oestrogen-treated 12-day old chick.

### 4.3.2. RCA in OOC and TOC

Figures 4.2a and 4.2b show the RCA of OOC infected with seven strains of IBV at an input dose of 4.0 $\log_{10}$ $CD_{50}$ per ml. All the isolates caused oviduct ciliostasis by day 5 p.i. Following an input dose of 2.0 $\log_{10}$ $CD_{50}$ (Figures 4.3a and 4.3b), the ciliary activity ceased by day 6 p.i.

Figures 4.4a and 4.4b show the RCA of TOC infected with seven strains of IBV at an input dose of 4.0 $\log_{10}$ $CD_{50}$. All caused ciliostasis by day 3 p.i. except strain G which took until day 4 p.i. Similar results were seen after an input dose of 2.0 $\log_{10}$ $CD_{50}$ (Figures 4.5a and 4.5b).

**Figure 4.1** Precociously-induced oviducts in an oestrogen-treated 12-day old SPF chick. This chick had both right and left oviducts developed.

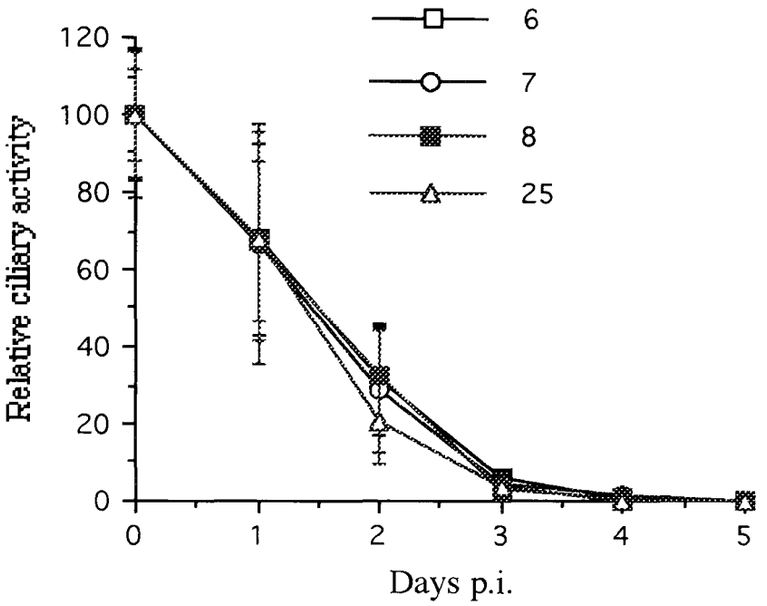


**Figure 4.2a** RCA of OOC infected with IBV strains at an input dose of 4.0 $\log_{10}$ $CD_{50}$.

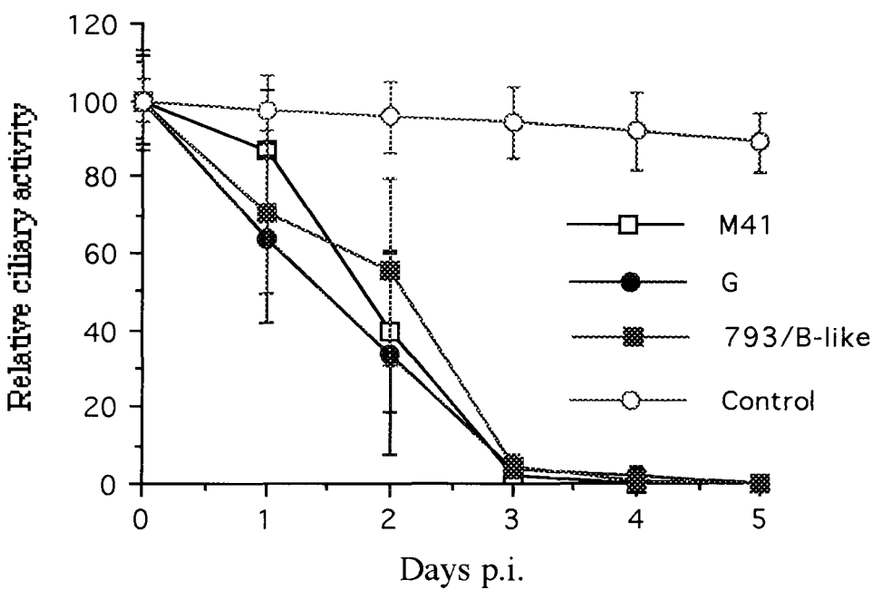


**Figure 4.2b** RCA of OOC infected with IBV strains at an input dose of 4.0 $\log_{10}$ $CD_{50}$.

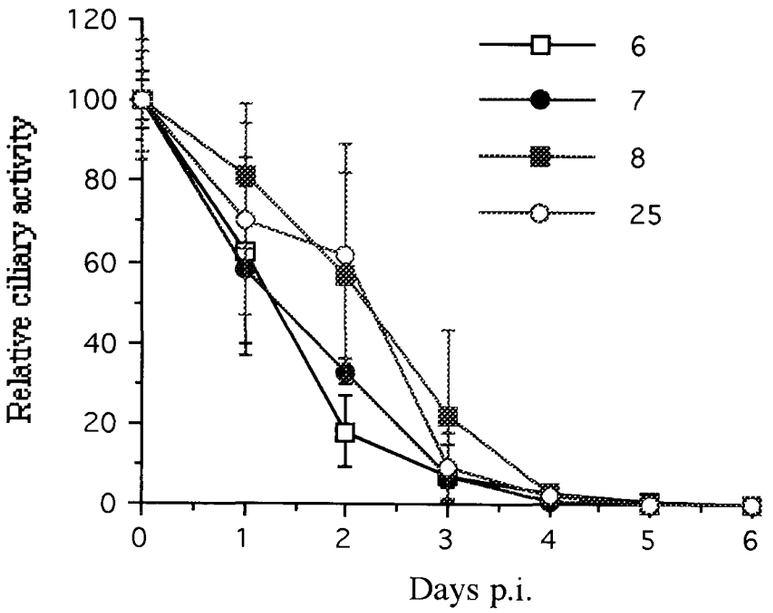


**Figure 4.3a** RCA of OOC infected with IBV strains at an input dose of 2.0 $\log_{10}$ $CD_{50}$.

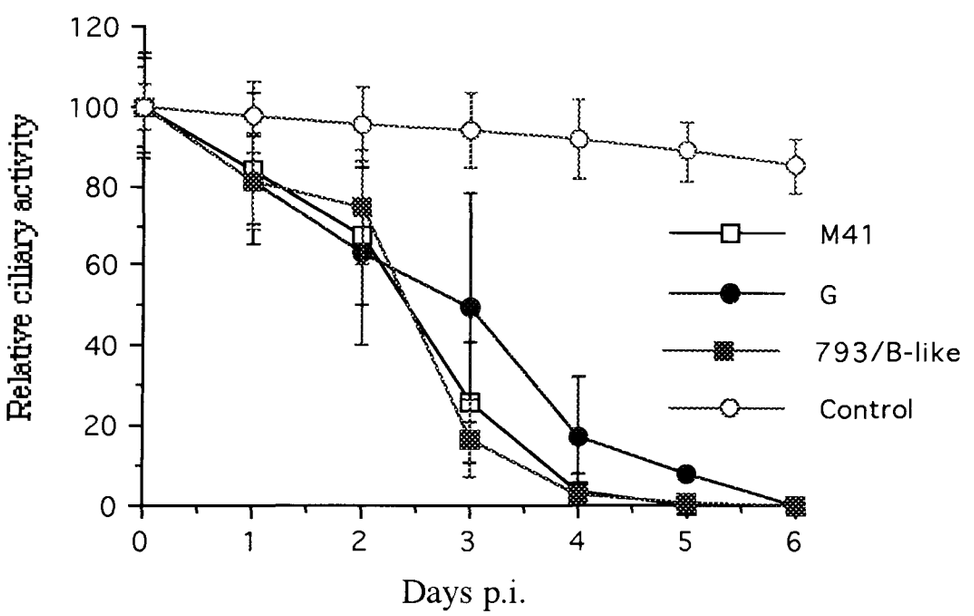


**Figure 4.3b** RCA of OOC infected with IBV strains at an input dose of 2.0 $\log_{10}$ $CD_{50}$.

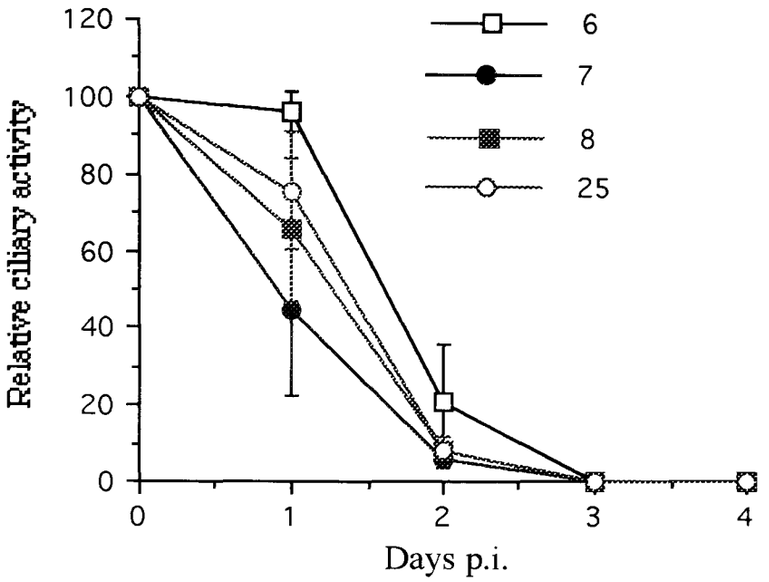


**Figure 4.4a** RCA of TOC infected with IBV strains at an input dose of 4.0 $\log_{10}$ $CD_{50}$.

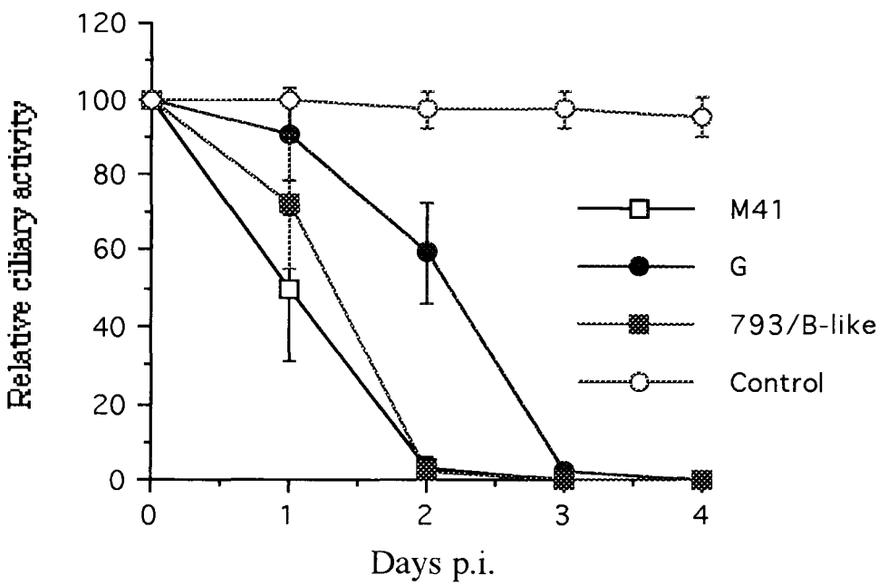


**Figure 4.4b** RCA of TOC infected with IBV strains at an input dose of 4.0 $\log_{10}$ $CD_{50}$.

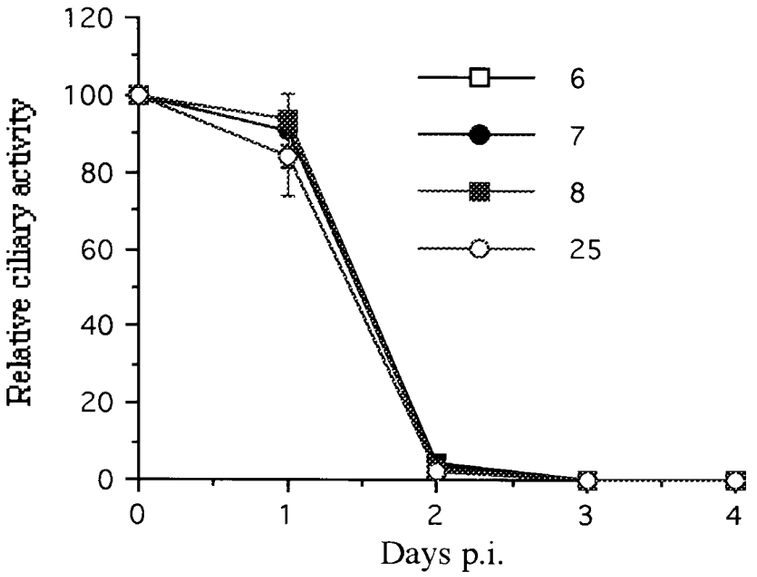


**Figure 4.5a** RCA of TOC infected with IBV strains at an input dose of 2.0 $\log_{10}$ $CD_{50}$.

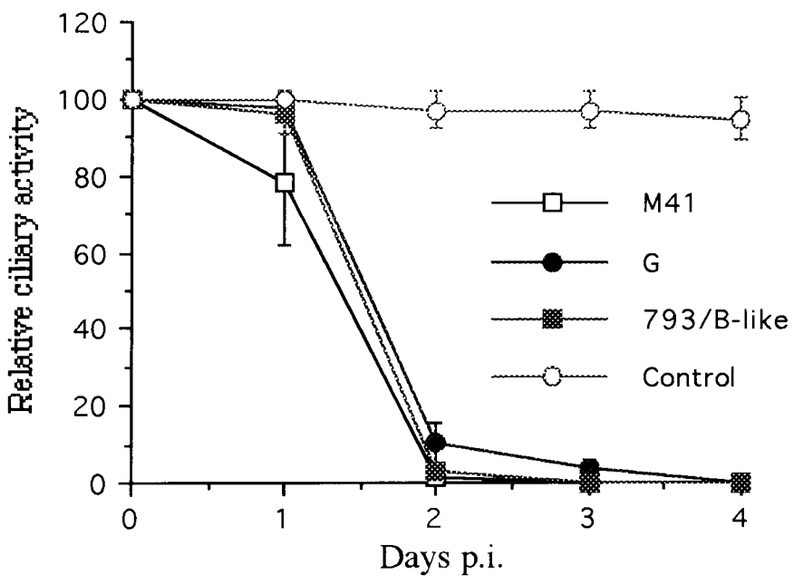


**Figure 4.5b** RCA of TOC infected with IBV strains at an input dose of 2.0 $\log_{10}$ $CD_{50}$.

Table 4.2 shows the time taken (in days) for a 50% reduction in RCA of OOC and TOC. All strains showed broadly similar virulence after both input doses. However, in OOC at a dose of 2.0 $\log_{10}$, the time taken for a 50% reduction in RCA was the longest for strain G and shortest for strains 6 and 7. At the higher dose, the times varied from 1.4 to 2.1 days. In TOC, after a dose of 4.0 $\log_{10}$, the time taken for 50% reduction in RCA was shortest for strains 7 and M41 and longest for strain G, while after the lower dose, all strains produced similar results.

**Table 4.2. Time taken in days for IBV strains to cause a 50% reduction in RCA of OOC or TOC.**

| IBV strains | OOC $10^4$ (n=18) | OOC $10^2$ (n=10) | TOC $10^4$ (n=10) | TOC $10^2$ (n=10) |
|---|---|---|---|---|
| 6 | 1.5 | 1.3 | 1.6 | 1.5 |
| 7 | 1.4 | 1.3 | 0.9 | 1.4 |
| 8 | 1.5 | 2.2 | 1.2 | 1.5 |
| 25 | 1.4 | 2.2 | 1.3 | 1.4 |
| M41 | 1.8 | 2.4 | 1.0 | 1.4 |
| G | 1.4 | 3.0 | 2.0 | 1.5 |
| 793/B-like | 2.1 | 2.4 | 1.3 | 1.5 |

### 4.3.3. IF staining of OOC

Figure 4.6 illustrates IBV-containing epithelial cells in OOC. Table 4.3 shows the mean fluorescence scores for oviducts infected with 4.0 $\log_{10}$ $CD_{50}$ of virus. Although all strains were capable of infecting oviducts, their mean fluoresence scores were variable and on days 1 - 3 p.i., they did not correlate with RCA. However, on days 4 and 5 p.i., the mean fluoresence scores of all strains tested were less than 1.0 which corresponded with more than 95% reduction in RCA or complete cessation of ciliary activity by this time.

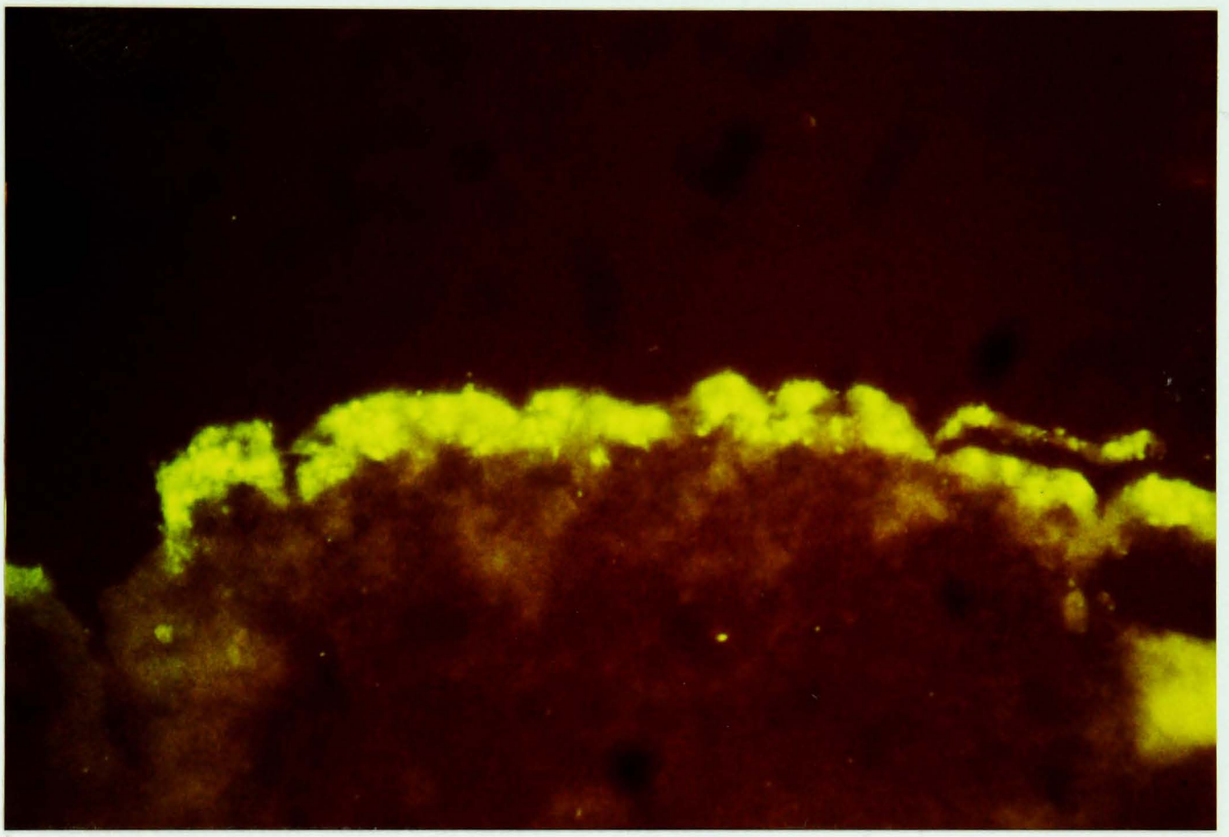

**Figure 4.6** IF staining of a section of OOC showing viral antigens of IBV strain 6 in epithelial cells on day 3 p.i. Magnification x125

**Table 4.3. Mean IF scores for OOC after infection with IBV strains.**

| Virus strain | Mean IF scores on days p.i. 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 6 | 2.0 | 2.83 | 1.17 | 0.67 | 0 |
| 7 | 3.0 | 2.33 | 0.83 | 0.50 | 0.33 |
| 8 | 1.67 | 1.17 | 2.5 | 0.33 | 0.33 |
| 25 | 0.33 | 2.0 | 1.67 | 0.33 | 0.67 |
| M41 | 2.33 | 1.67 | 0.33 | 0.33 | 0 |
| G | 0.33 | 3.0 | 0.5 | 0.33 | 0 |
| 793/B-like | 2.83 | 1.67 | 0.67 | 0.17 | 0 |
| Control | 0 | 0 | 0 | 0 | 0 |

### 4.3.4. Virus titres in OOC.

All seven strains were capable of growing in OOC at doses of 2.0, 4.0 or 6.0 $\log_{10}$ $CD_{50}$. Figure 4.7 depicts the titres in the supernatants of OOC infected with 2.0 $\log_{10}$ $CD_{50}$ of each strain. Strain 6 produced the highest titre on day 3 p.i. which reflects the shortest time taken by this virus for producing a 50% reduction in RCA.

### 4.3.5. Calmodulin assay for tissue damage.

Figure 4.8 shows the CAM concentrations in the supernatant fluids of OOC infected with 4.0 $\log_{10}$ $CD_{50}$ of virus on days 1, 3 and 5 p.i. The validity of the assay was established by using CAM standards and a CAM antagonist. All strains tested had significantly higher CAM concentrations in the supernatants than control cultures only on day 5 p.i.(student's t-test, $P<0.05$). The levels of CAM produced by the IBV strains were broadly similar. However, strains 6 and M41 produced higher levels of CAM while strains 7, 25 and G produced relatively lesser amounts. Viruses 8 and 793/B-like were intermediate.

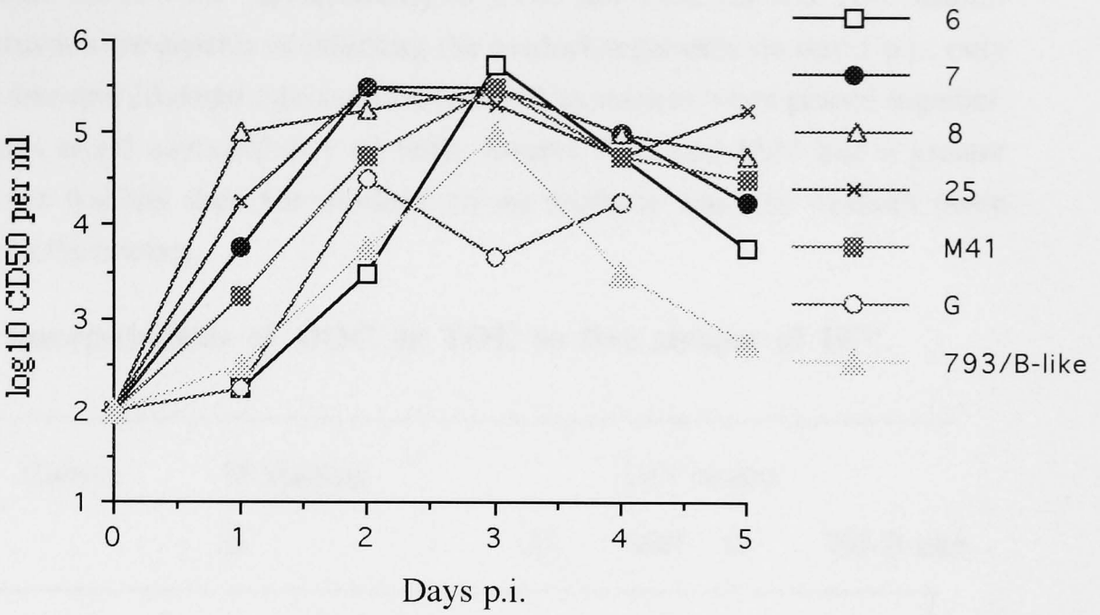


**Figure 4.7.** Titres of IBV strains in supernatants of OOC after infection with 2.0 $\log_{10}$ $CD_{50}$.

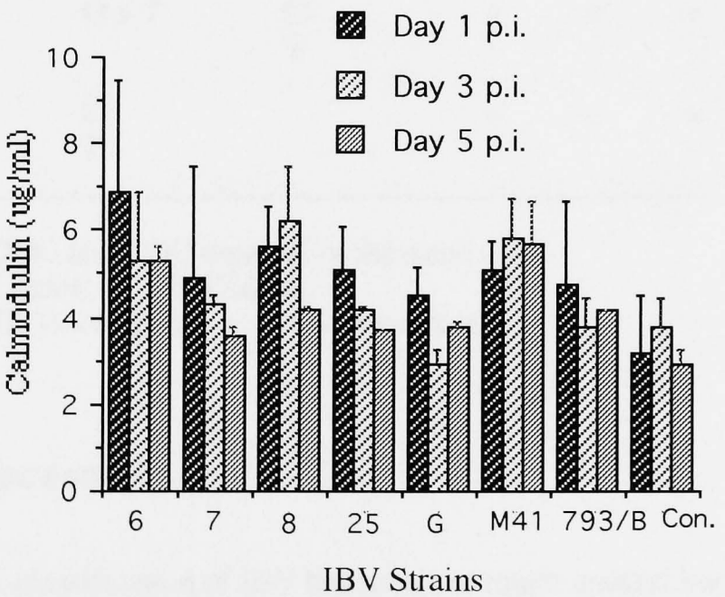


**Figure 4.8.** CAM concentrations in supernatants of OOC after infection with IBV strains.

### 4.3.6. *In vitro* susceptibility of OOC and TOC.

Table 4.4 shows the *in-vitro* susceptibility of OOC and TOC for five IBV strains. Though all viruses were capable of infecting the oviduct separately on day 1 p.i., only strains 793/B-like and 25 could infect oviduct as well as trachea when placed together, which indicates equal susceptibility of both. Strains 7, G and M41 had a greater predilection for trachea than for oviduct. In no instance was the oviduct more susceptible than the trachea.

**Table 4.4. Susceptibilities of OOC or TOC to five strains of IBV.**

| Days p.i. | Culture | IF staining in | IBV strains 7 | 25 | M41 | G | 793/B-like |
|---|---|---|---|---|---|---|---|
| 1 | O+T | O | - | + | - | - | + |
| | | T | + | + | + | + | + |
| | O | | + | + | + | + | + |
| | T | | + | + | + | + | + |
| 2 | O+T | O | + | + | + | + | + |
| | | T | + | + | + | + | + |
| | O | | + | + | + | + | + |
| | T | | - | + | + | + | + |
| 3 | O+T | O | + | + | + | + | + |
| | | T | - | - | - | + | - |
| | O | | + | + | + | + | + |
| | T | | - | - | - | - | - |

O + T - OOC and TOC together in the same tube.
O - OOC alone; T - TOC alone.
+: specific fluorescence, -: no fluorescence.

## 4.4. DISCUSSION

Serotypic classification of IBV has received much interest but the relationship between serotype and pathogenicity is not well understood (Avellanda et al., 1994). With the constant emergence of new serotypes and variants (Cook,1984; Cook and Huggins, 1986), it is important to know the pathogenicity of the challenge isolates in order to choose an efficacious vaccine. Cook and Huggins (1986) noted that the difficulty in quantifying severity of respiratory infection caused by IBV has always been a problem

in studying its pathogenicity. The development of a dual IBV and *E.coli* infection model by Smith et al. (1985) has enabled the virulence assessment of IBV isolates *in-vivo*, using mortality and/or lesion scores in infected birds as measures of virulence (Cook et al., 1986; Avellanada et al., 1994). However, no *in-vitro* method is available to assess the virulence of IBV strains. The present study was designed to compare *in-vitro*, the virulence and susceptibility for the oviduct of several IBV strains belonging to different serotypes.

Peters et al. (1979) have shown that OOC prepared from different regions of the oviduct or differing ages of birds did not significantly alter the minimum infectious dose of the H52 vaccine for the tissue. Moreover, IBV is capable of causing infection and permanent damage to oviducts even in day-old chicks when oviducts are in a highly undifferentiated and immature state (Crinion et al.,1971a, Jones and Jordan, 1970). Thus the use of OOC prepared from oestrogen-treated chicks appears to be a simple alternative to the constraints of maintaining chicks until their oviducts become bigger and differentiated for such pathogenicity trials.

All the IBV strains tested caused oviduct and tracheal ciliostasis by days 5 and 3 p.i. respectively. The results of oviduct ciliostasis correlated well with the complete absence or the presence of only a few fluorescing cells in the epithelium at this time. Pradhan et al (1984), using stationary vessels obtained oviduct ciliostasis by 6 days p.i., using a field isolate belonging to the Massachusetts serotype. The M41 virus used in this study caused ciliostasis by day 5 p.i. This slight difference could be attributed to better culture conditions and constant rolling of OOC, allowing optimal conditions for infection.

The quantitative measurement of ciliary activity has been used for comparing the virulence of several mycoplasma strains (McGee et al.,1976; Bermudez et al., 1990; Stadlander et al 1991). In the present study, the time taken for 50% reduction of RCA was used as one of the criteria for virulence. Using this, it was found that strain 6 (serotype D207) was the most virulent for oviducts even at a low input dose while G was least. Correspondingly, strain 6 had the highest titre in the supernatant of OOC at day 3 p.i. of any virus tested. Though strain 6 was the most virulent for oviduct cilia, it was the least for tracheal cilia together with strain G. Strain 7 (serotype D3896) and M41 were the most pathogenic for tracheal cilia. Strain G has been known to be an enterotropic variant with greater predilection for and persistence in the gut than trachea (El Houadfi et al.,1986).

All the seven IBV strains used in this study, have been shown to be associated with a drop in egg production and an increase in poor egg quality in infected chickens (Crinion

et al., 1971b; Cook,1984; El Houadfi et al., 1991; Parsons et al., 1992). Crinion and Hofstad (1972a) have shown that IBV strains Connecticut and Iowa 609 were not pathogenic for the oviducts. These two strains were not used in this study. It would be interesting to test these two strains in this model system.

Although, all the strains grew in OOC incubated separately, on day 1 p.i. only two (25 and 793/B-like) had an equal predilection for both oviduct and trachea in cultures incubated together. No fluoresence was seen on day 3 p.i. in the epithelium of tracheal cultures infected alone or (with one exception) together with oviduct. By this time, all ciliated epithelial cells in the trachea would have been lost (see Figures 4.4a and 4.4b).

Crinion and Hofstad (1972b) showed that repeated embryo passage reduced the pathogenicity of strain M41 for the oviduct. However, the M41 used here had had numerous TOC passages and it was still pathogenic for OOC. This discrepancy may be attributed to the different laboratory host systems and presumably repeated passage in TOC, simulating natural host tissue, causes little or no attenuation.

In the part of this study relating to CAM, concentrations of the protein were analysed only in the medium supernatants, where they were expected to increase due to epithelial cell damage. A corresponding decrease in the CAM concentrations of oviduct tissue homogenates was not shown. The CAM assay revealed significant differences between infected and control values only on day 5 p.i., though ciliary activity was decreased from day 1 p.i. itself. This could have been attributable to some degenerative damage to the epithelium in control cultures, even though ciliary activity was not markedly decreased. Nonetheless, the CAM assay showed that strains 6 and M41 were the most virulent for oviducts followed by strains 8 and 793/B-like. Strains 7, G and 25 were relatively less virulent.

Thus the time taken for 50% reduction in RCA and concentrations of CAM in the supernatants of infected OOC might be able to differentiate pathogenicity of IBV isolates for the oviducts. However, before proposing the use of this model for pathotyping IBV *in-vitro,* further studies using strains belonging to different serotypes and perhaps different strains of the same serotype are required .

# CHAPTER 5

## GROWTH OF INFECTIOUS BRONCHITIS VIRUS VACCINES IN OVIDUCTS DERIVED FROM OESTROGEN-TREATED CHICKS AND EMBRYOS.

**CONTENTS** **PAGE NUMBER**

# CHAPTER 5

## GROWTH OF INFECTIOUS BRONCHITIS VIRUS VACCINES IN OVIDUCTS DERIVED FROM OESTROGEN-TREATED CHICKS AND EMBRYOS.

### 5.1. INTRODUCTION

Replication of IBV in the epithelium of the oviduct of the mature female chicken results in decreased egg production and quality (Sevoian and Levine, 1957; McDougall, 1968). The damage may be more severe and permanent if birds are infected soon after hatching (Crinion et al., 1971a; Jones and Jordan, 1972). To date, studies involving the virulence of different strains of IBV for the chicken oviduct have been rare (McMartin, 1993). Evidence for differences in virulence between strains of IBV for the oviduct was reported by Crinion and Hofstad (1972a). The virulence of seven field strains of IBV for the chicken oviduct was compared in Chapter 4, using OOC and scoring of ciliary activity as one of the criteria for assessment of damage to the oviduct ciliated epithelium.

Live attenuated IBV vaccines are extensively used to protect young chicks against IBV. However, very little information is available regarding the pathogenicity of vaccine viruses for the chicken oviduct. This is probably related to the difficulty in conducting tests like IF staining, histopathology or virus isolations on the small amount of oviduct tissue normally present in young female chicks. Despite this difficulty, Crinion and Hofstad (1972b) have shown, using histopathology and IF studies, that repeated embryo passage of the Massachusetts strain of IBV reduced its virulence for the chicken oviduct.

Current methods employed for attenuation of IBV include serial passages in chicken embryos or cell cultures (Larose and van Roekel, 1961; Gelb and Cloud, 1983; Klieve and Cumming, 1988; Gelb et al., 1991; Wakenell et al., 1995). The degree of attenuation is assessed by inoculation of susceptible chicks and monitoring for clinical disease, histopathological lesions or virus isolations (Gelb and Cloud, 1983). Scoring of tracheal ciliary activity in infected poults has also been used to measure the level of attenuation of TRTV (Cook et al., 1989) but no *in-vitro* method is available for assessment of attenuation of IBV.

TOC prepared from 19- or 20-day old chicken embryos, are routinely used for the cultivation and assay of IBV (Cook et al., 1976). In this study, an attempt was made to prepare OOC using oviducts derived from chicken embryos treated with oestrogen preparations.

The purpose of the present study, therefore, was three-fold:
1) To compare the ability of six commercial vaccines to grow in the OOC and TOC.
2) To validate an *in-vitro* method for assessment of attenuation of IBV.
3) To prepare OOC from oestrogen -treated embryo oviducts and to determine the value of such cultures in studying IBV.

## 5.2. MATERIALS AND METHODS

### 5.2.1. Chicks

SPF eggs obtained from a commercial source were hatched in our laboratory and the chicks maintained in strict isolation throughout the study as described in Chapter 3.

### 5.2.2. Vaccines

Six commercial live vaccines were used after a single passage in 10-day old embryonated SPF chicken eggs via the allantoic cavity. The allantoic fluid was harvested after 48 hours and used as the source of inoculum. The vaccine viruses used in these experiments and their serotypes are listed below in Table 5.1.

**Table 5.1. Commercial IBV vaccines used.**

| Vaccine | Serotype |
|---|---|
| A | Massachusetts - H52 |
| B | Massachusetts - H120 |
| C | Massachusetts - MM |
| D | Massachusetts - Ma 5 |
| E | D274 |
| F | D1466 |

### 5.2.3. Viruses

Five field strains of IBV were used and their details are shown in Table 5.2.

**Table 5.2. Field strains of IBV used.**

| IBV | Serotype | Reference |
|---|---|---|
| 6 | D207 | Cook (1984) |
| 7 | D3896 | Cook (1984) |
| M41 | Massachusetts | Ambali and Jones (1991a) |
| G | (undesignated) | El Houadfi et al. (1986) |
| 793/B-like | (undesignated) | Chapter 8 |

### 5.2.4. Antiserum

Hyperimmune serum to IBV strain G was raised in adult SPF cockerels according to the method described in Chapter 3 and used for the detection of group specific IBV antigen in IF tests.

### 5.2.5. Tracheal organ cultures

TOC were prepared from 19-day old embryonated SPF chicken eggs or from oestrogen-treated embryos or chicks, following the procedure described in Chapter 3.

### 5.2.6. Oviduct organ cultures

SPF chicks were inoculated with 1mg (0.2 ml) of oestradiol-benzoate (Intervet, UK) subcutaneously on days 1, 5 and 9 post-hatch. Two or three days later, oviducts were collected from female chicks post-mortem. OOC were prepared according to the method described in Chapter 3.

### 5.2.7. Virus titrations

Serial dilutions of IBV vaccines or field strains were inoculated in to groups of 3 or 5 tubes of OOC or TOC. Three different parameters were used to determine endpoints.

**5.2.7.1. Ciliostatic dose ($CD_{50}$)**: Stasis of oviduct or tracheal cilia was used as the criterion for the presence of virus in the conventional way (Cook et al., 1976). Following

inoculation of cultures, tubes were incubated in a roller drum and examined daily for up to six days for the presence of ciliary activity. Tubes showing 100% ciliostasis were considered positive for the presence of virus. Ciliostasis, a manifestation of cell damage thus indicates the pathogenic nature of the virus.

**5.2.7.2. Immunofluorescence infective dose ($IFID_{50}$)**: 1mm thick sections of oviduct or trachea were inoculated with serial dilutions of IBV vaccines or field strains. Two days after infection, tissues were processed for IF staining as described in Chapter 3. IF was performed using hyperimmune serum against IBV and affinity-purified anti-chicken IgG-FITC-labeled conjugate (Sigma) as described in Chapter 3. The presence of virus-antigen in oviduct or tracheal sections was indicated by specific fluorescence in the epithelium. IF staining shows the presence of viral antigens but not necessarily the infectious or pathogenic nature of the virus.

**5.2.7.3. Organ culture infective dose ($OCID_{50}$)**: This was determined according to the method of Darbyshire et al. (1976). Tenfold dilutions of virus were prepared and each dilution used to infect six replicate explants of OOC or TOC. Twenty four hours after infection, the medium was changed. Assays of infectivity were done in TOC from the pooled fluid harvest of pairs of replicates collected after a further 24 hours. When a titre of $\geq 2.3$ $\log_{10}$ $CD_{50}$/ml of virus was demonstrable in the pooled fluid harvest from any pair of replicates, this was considered to indicate that significant infection of those tissue explants had occurred and was accordingly scored as being positive. The $OCID_{50}$ gives an indication of the replication and production of infectious virus. It is likely to be more sensitive than virus infectivity measured by straightforward ciliostasis because it involves an extra passage of the supernatants of infected cultures.

Titres for all three procedures were calculated according to the method of Reed and Muench (1938) and expressed as $\log_{10}$ titres in 0.1 ml.

### 5.2.8. Experimental designs

#### 5.2.8.1. Titration of vaccines in TOC from normal and oestrogen-treated embryos and chicks

Six commercial vaccines were titrated in TOC prepared from 19-day old chick embryos, 20-day old oestrogen-treated chicken embryos or 12-day old oestrogen-treated chickens to exclude any adverse effects of oestrogen treatment on virus titres.

#### 5.2.8.2. Titration of vaccines and virulent IBV strains in TOC and OOC

Virus titres of six commercial vaccines and five virulent IBV strains were compared in TOC and OOC systems using ciliostasis as indicator for the prescence of virus ($CD_{50}$).

#### 5.2.8.3. Comparison of end-point parameters

Virus titres of six commercial vaccines and five IBV strains were compared using ciliostasis, IF or organ culture infectivity as endpoints ($CD_{50}$, $IFID_{50}$ or $OCID_{50}$).

#### 5.2.8.4. Inoculation of embryos with oestrogen

In an attempt to adapt the chicken OOC model system to embryos, different preparations of oestrogen (oestradiol benzoate, oil based, Intervet) or ß-oestradiol (water-soluble, Sigma) were inoculated through different routes and at different ages of the embryos. The approximate weight of the embryo oviducts and the presence of cilia was determined on day 20 of incubation for each regimen of inoculations.

The efficacy of the *in-ovo* model of preparing OOC was analysed by comparing the $IFID_{50}$ for six commercial vaccines in OOC prepared from chickens and those prepared from oestrogen-treated embryos.

### 5.2.9. Statistical analysis

All the experiments were repeated twice and the results compared for significant differences using the students t-test ($p<0.05$).

## 5.3. RESULTS

### 5.3.1. Titration of vaccines in TOC from normal and oestrogen-treated embryos and chicks

No significant differences were recorded in titres determined using ciliostasis as endpoints for six commercial vaccines in TOC prepared from 19-day old chicken embryos, 20-day old oestrogen-treated chicken embryos or 12-day old oestrogen-treated chicks (Figure 5.1).

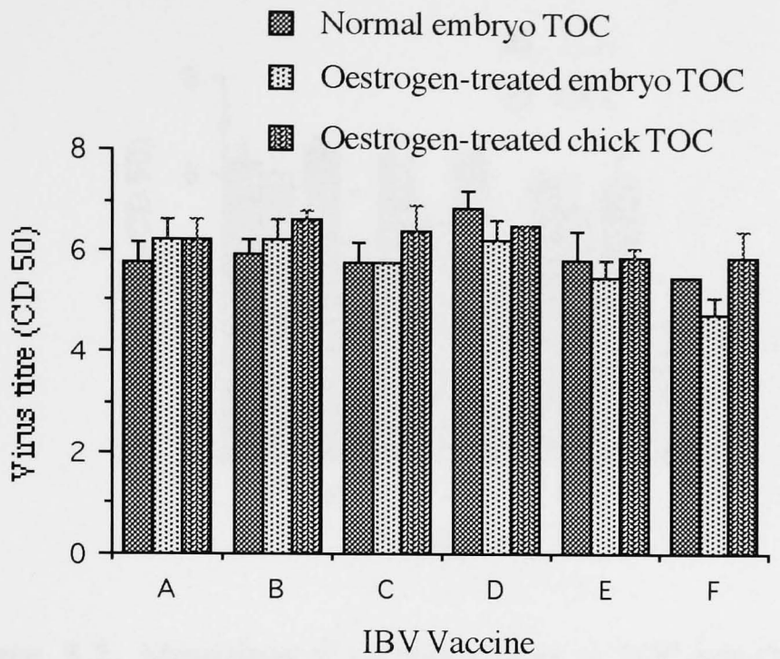


**Figure 5.1.** Mean titres of vaccine viruses in conventional TOC, oestrogen-treated embryo and chicken TOC. Vertical bars represent standard deviations.

### 5.3.2. Titration of vaccine and virulent IBV in TOC and OOC

Of the six commercial vaccines tested, three vaccines (B, D and F) were found to grow to significantly lower titre in OOC compared to TOC, using ciliostasis as endpoints (Figure 5.2). Of the five virulent strains of IBV tested, only the enterotropic strain G was found to grow significantly less well in OOC as compared to TOC (Figure 5.3).

### 5.3.3. Comparison of endpoint parameters

When the virus titres for six commercial vaccines were determined using ciliostasis, IF or organ culture infectivity as endpoints, it was found that two of the three vaccines which grew poorly in OOC (B and D) had significantly higher titres as measured by $IFID_{50}$ and $OCID_{50}$ as compared to $CD_{50}$ (Figure 5.4). Similar results were obtained when the vaccines were grown in TOC (Figure 5.5), except that the $OCID_{50}$ of vaccine B was not significantly different from the $CD_{50}$.

Comparison of titres of the five virulent viruses using the three different endpoints revealed no significant differences between them in OOC (Figure 5.6) or TOC (Figure 5.7).

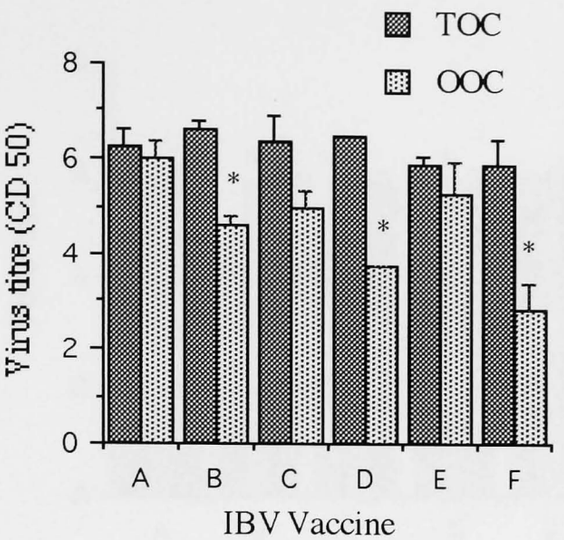


**Figure 5.2.** Mean titres of vaccine viruses in TOC and OOC. * represents significant differences in titres compared to TOC.

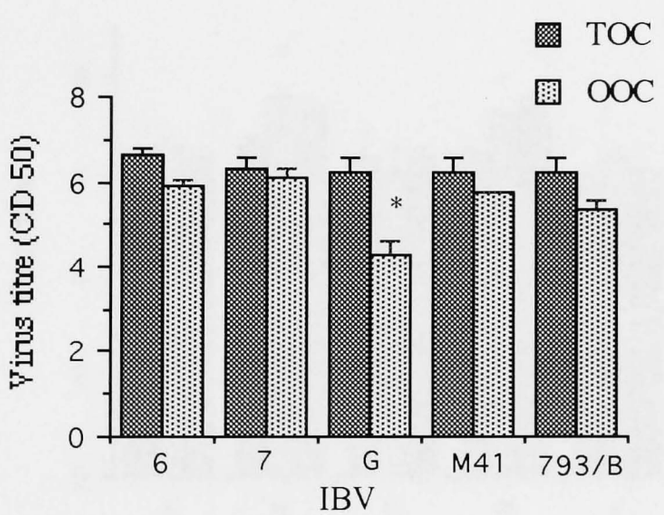


**Figure 5.3.** Mean titres of IBV in TOC and OOC. * represents significant differences in titres compared to TOC.

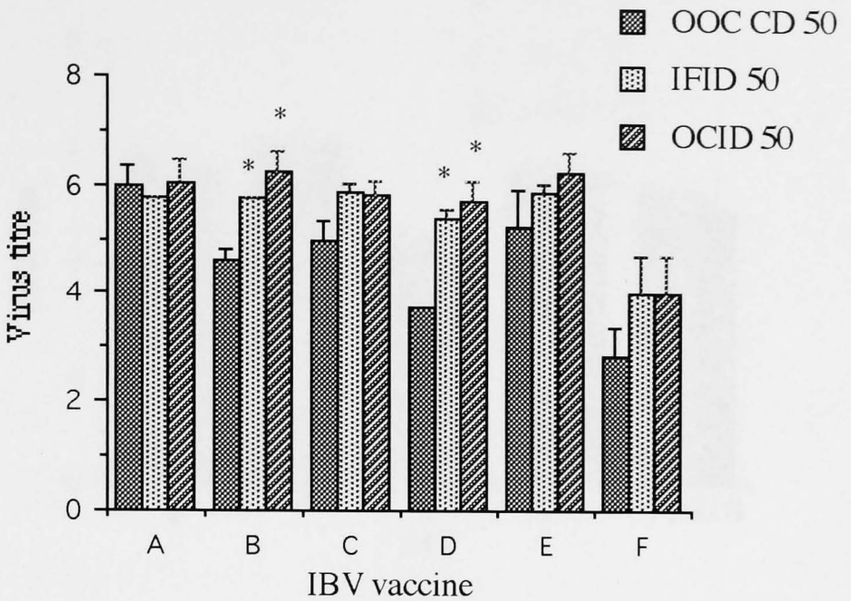


**Figure 5.4.** Mean titres of vaccine viruses in OOC determined by three different endpoints. * indicates significant differences in titres compared to $CD_{50}$.

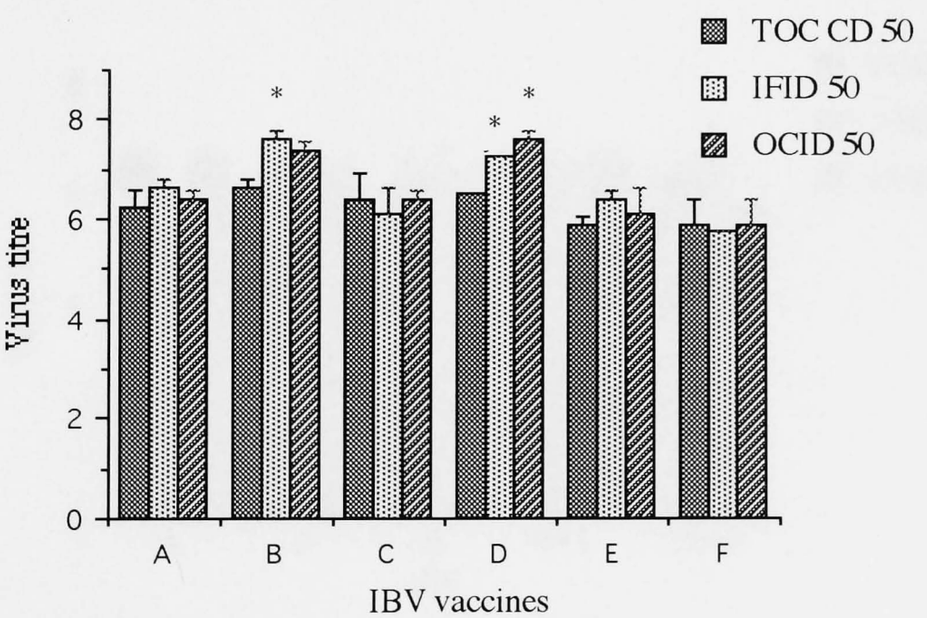


**Figure 5.5.** Mean titres of vaccine viruses in TOC determined by three different endpoints. * indicates significant differences in titres compared to $CD_{50}$.

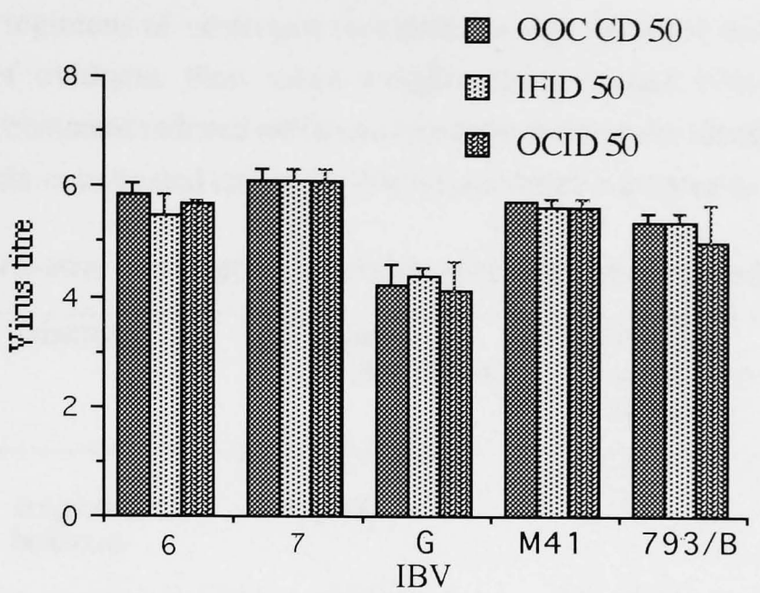


**Figure 5.6.** Mean titres of five IBV in OOC determined by three different endpoints.

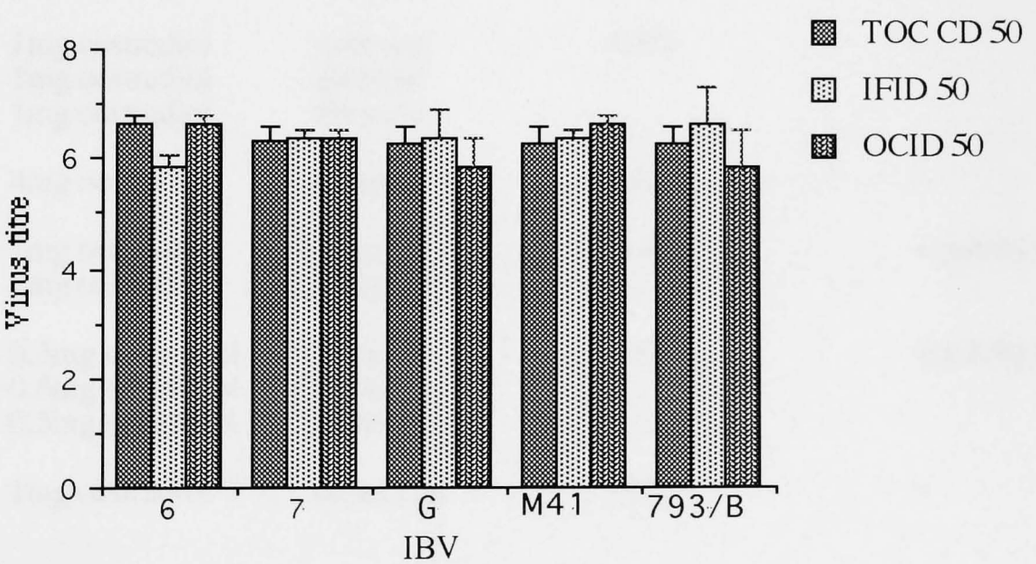


**Figure 5.7.** Mean titres of five IBV in TOC determined by three different endpoints.

### 5.3.4. Inoculation of embryos with oestrogen

The different regimens of oestrogen inoculations into embryos did not cause dramatic enlargement of oviducts, their mean weights being around 0.01g only (Table 5.3). Nevertheless, treatment induced sufficient enlargement to make identification easy, which was not possible in untreated embryos. When inoculation was done at 5 days of age the oil-

**Table 5.3. *In-ovo* oestrogen administration for preparation of OOC**

| Age of embryo (days) | Treatment | Route of inoculation | Oviduct weight (g) (approx) | Cilia |
|---|---|---|---|---|
| 5 | 1mg oestradiol benzoate | yolk-sac | - | - |
| 14 | 1mg oestradiol benzoate | allantoic | 0.01 | - |
| 11<br>14 | 2mg oestradiol<br>1mg oestradiol | allantoic<br>allantoic | 0.02 | +(patchy) |
| 11<br>14 | 1mg oestradiol<br>1mg oestradiol | allantoic<br>allantoic | 0.01 | - |
| 5<br>11 | 1mg oestradiol<br>1mg oestradiol | yolk-sac<br>allantoic | 0.01 | - |
| 5<br>11<br>14 | 1mg oestradiol<br>1mg oestradiol<br>1mg oestradiol | yolk-sac<br>allantoic<br>allantoic | 0.02 | - |
| 14 | 4mg oestradiol | allantoic | 0.008 | - |
| 14<br>18 | 1mg oestradiol<br>1mg oestradiol | allantoic<br>broad end | 0.025 | +(patchy) |
| 14<br>16<br>18 | 0.5mg oestradiol<br>0.5mg oestradiol<br>0.5mg oestradiol | allantoic<br>allantoic<br>allantoic | 0.025 | +(patchy) |
| 18 | 1mg oestradiol | broad end | 0.01 | - |

based oestrogen preparation killed all the embryos. None of the schedules induced uniform ciliary activity in the oviducts. However, IF staining confirmed that these oviducts were capable of being infected by IBV. Hence, an easy schedule of inoculating embryos at 18

days of incubation and collecting oviducts on day 20 from female embryos was followed for subsequent trials.

When the vaccine titres determined in OOC prepared from oestrogen-treated chicken or embryo oviducts using IF as endpoints were compared, it was seen that five of the six vaccine viruses grew significantly less well in embryo OOC than in chicken OOC. Vaccine F, belonging to D1466 serotype, did not show any significant differences in titres obtained from chicken or embryo OOC (Figure 5.8).

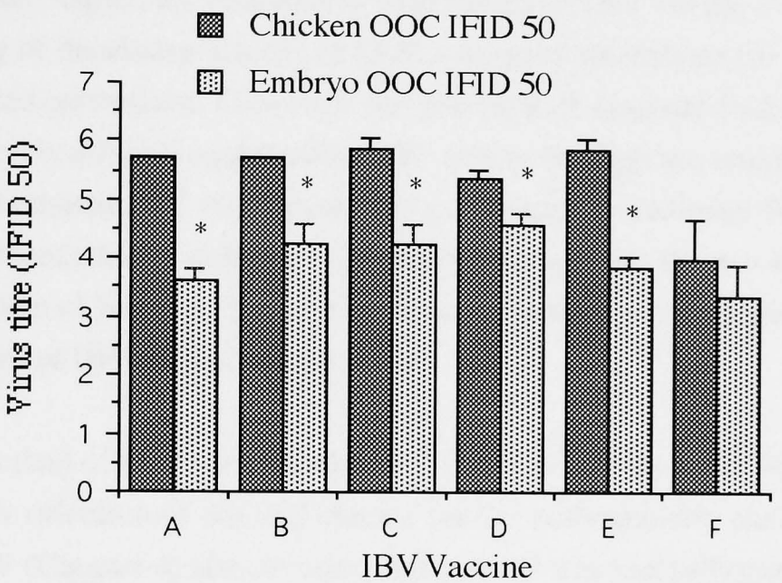


**Figure 5.8.** Mean $IFID_{50}$ titres of vaccine viruses in OOC prepared from oestrogen-treated chickens or embryos. * indicates significant reduction in titres compared to those determined in OOC from oestrogen-treated chicks.

## 5.4. DISCUSSION

The dearth of information on the interaction between IBV and oviducts may be due to the practical constraints of maintaining birds till their oviducts become mature and differentiated for conducting such experiments. The use of oestrogen treatment to induce precocious development of oviducts may provide an alternative to such long-term experiments. It is known that IBV is capable of infecting oviducts from day-old and adult chickens alike, irrespective of the stage of differentiation of this tissue (Crinion et al., 1971a; Jones and Jordan, 1971). However, the damage is more severe and permanent if young chicks are

infected. Peters et al. (1979) have shown that normal oviducts obtained from different ages of chicken and different regions of the oviduct were equally susceptible to the H52 vaccine virus. This indicates that oviducts from oestrogen-treated chicks may be a good substrate for virus infection despite their artificially induced growth. In the present study it was also seen that oestrogen treatment of chicks or embryos did not alter the titres of six commercial vaccines significantly when titrated in TOC. Similar studies could not be performed with oviducts in age-matched oestrogen-treated and untreated chicks since OOC could not be prepared from untreated 2-week old chicks.

In the previous chapter, the virulence of field strains of IBV for the oviducts was assessed using scoring of the ciliary activity in OOC, as one of the criteria for assessing damage to oviduct ciliated epithelium. However, the presence of mucosal folds in OOC makes the scoring relatively difficult compared to TOC, where the rings are circular and supported by cartilage. Determination of virus titres, using ciliostasis as indicator for presence of virus, requires differentiation of cultures with ciliary beating from those without, rather than to assess the extent of beating. Hence virus titres were used to assess growth of vaccine and virulent strains of IBV in these experiments.

Jones and Ambali (1987) reported that IBV strain G did not cause severe damage to the oviducts after infection of day-old chicks. Earlier pathogenicity studies with seven field strains of IBV (Chapter 4) also revealed that strain G was less pathogenic for oviducts. The present work also revealed that this strain grew significantly less well in OOC than TOC, using ciliostasis as the endpoint. Thus, the use of precocious oviducts may help determine relative tropisms of IBV strains for the reproductive tract.

Three of the six commercial vaccines tested grew significantly less well in OOC compared to TOC. One explanation for this difference could be that OOC are more refractory than TOC. Another could be that ciliostasis is related to the pathogenic ability of a virus and may not be a true indicator of the actual virus titre of attenuated vaccines. Two of these three vaccines, H120 and an M41-derived vaccine (Ma5) are highly attenuated and can be safely given to day-old chicks. To check the hypothesis we used two other endpoints for determining virus titres, namely $IFID_{50}$ and $OCID_{50}$. Since IF detects only virus specific antigen, $OCID_{50}$ was included to detect presence of infective virus.

Our hypothesis was correct for two of the three poorly growing viruses in OOC, wherein we found that virus titres determined by $IFID_{50}$ or $OCID_{50}$ were significantly greater than

those determined by $CD_{50}$. This would suggest that there is more virus actually replicating in these tissues than that evidenced by ciliostasis. This is what one would expect from an attenuated and safe vaccine i.e. the vaccine virus actually multiplies in the tissues (and induces an immune response) but causes less damage. Thus we propose the use of differences between $IFID_{50}$ and $CD_{50}$ as a measure of attenuation of IBV vaccines. To validate this theory further we used five virulent viruses and found no significant differences in titres measured using the three different endpoints. This means that all the multiplying virus in each case was capable of causing ciliostasis.

This *in-vitro* method of determining degree of attenuation of vaccine viruses will be helpful for screening candidate viruses for vaccine production, although the *in-vivo* method will be the ultimate test for the safety of a vaccine. This technique does not give any information about the potency of a vaccine. Further investigations are needed to apply this method for other ciliostatic viruses like TRTV. Naylor and Jones (1994) have shown that the protective ability of the TRTV vaccine did not depend on a ciliostatic component.

Sufficient oviduct tissue is required to prepare OOC and the presence of cilia is needed to easily quantify the damage caused by IBV to oviducts. Hence the oviduct weight and ciliary activity were used as criteria for assessing the efficacy of various regimens of oestrogen inoculations in to the chicken embryos. The mean weight of oviducts in oestrogen-treated chicks was about 1g (Chapter 4) while that from oestrogen-treated embryos was approximately one-hundredth of this weight. Though some schedules induced ciliary activity, it was not uniform enough to be used as a measure of oviduct damage caused by IBV. However, IF staining confirmed that these embryo oviducts were still capable of being infected by the virus. Oviducts of normal day-old chicks are also capable of being infected *in-vivo* by IBV, even though they are in a highly undifferentiated state (Jones and Jordan, 1972).

In the present work, five out of six commercial vaccines grew significantly less well in OOC from embryos than in OOC from chicks. This may have been due to the smaller numbers of ciliated epithelial cells in the relatively undifferentiated tissue. Only the vaccine derived from strain D1466 had similar titres in both types of OOC. Even in OOC prepared from oestrogen-treated chickens, this vaccine virus was the most poorly growing and perhaps there were enough target ciliated epithelial cells even in embryo oviducts for this virus to multiply and produce similar titres.

# CHAPTER 6

## LOCAL ANTIBODY PRODUCTION IN THE OVIDUCT AND GUT OF HENS INFECTED WITH AN ENTEROTROPIC STRAIN OF INFECTIOUS BRONCHITIS VIRUS

**CONTENTS** **PAGE NUMBER**

# CHAPTER 6

# LOCAL ANTIBODY PRODUCTION IN THE OVIDUCT AND GUT OF HENS INFECTED WITH AN ENTEROTROPIC STRAIN OF INFECTIOUS BRONCHITIS VIRUS

## 6.1. INTRODUCTION

Immunisation studies with IBV have shown a lack of correlation between the levels of neutralising antibodies in the serum and resistance of chickens to infection (Hitchner et al., 1964; Raggi and Lee, 1965; Winterfield and Fadly, 1972). There is also considerable evidence that serological relationships among IBV isolates, determined by *in vitro* neutralisation tests have not been mirrored by the results of *in vivo* cross-immunity studies (Darbyshire, 1980; Darbyshire, 1985). Virus re-excretion has been shown to occur even in the presence of high titres of neutralising antibodies (Jones and Ambali, 1987). These findings indicate the importance of local or cell-mediated immunity in IBV infections.

The importance of locally-produced antibodies in the defence of mucosal surfaces against viral infections has been well documented. The trachea is a main target organ of IBV and local immunity in that organ has been demonstrated (Gomez and Raggi, 1974; Gillette, 1981; Nakamura et al., 1991). Dachryoantibodies (in tears) have also been implicated in protection against IBV (Davelaar et al., 1982).

Much of the economic importance of IBV is due to the decrease in egg production and quality caused by the pathological effects of the virus on the oviduct. Lymphocyte and plasma cell infiltration has been shown to occur in the oviducts of IBV-infected chickens by day 2 p.i. (Sevoian and Levine, 1957; Crinion et al., 1971a). Box and Ellis (1985) reported that oil emulsion vaccines given without prior attenuated vaccine priming did not confer adequate protection against a drop in egg production, though it produced good HI antibody responses. This phenomenon has been attributed to the lack of stimulation of local immunity by the killed vaccines. In mammals, the reproductive tract has been shown to be a component of the secretory immune system (Winter, 1982). Accumulated evidence suggests a possible role for local immunity in the chicken oviducts in response to IBV.

A variant IBV strain, IBVG, originally isolated from Morocco (El-Houadfi et al., 1986) has been found to be enterotropic by virtue of its greater persistence in the gut than in

the trachea (Jones and Ambali, 1987). Several strains of IBV have been isolated from the gut (Cook, 1984; Lucio and Fabricant, 1990) and explants of gut tissues were shown to support replication of IBV strains isolated both from respiratory tract and gut tissues (Bhattacharjee, 1994).

The present study deals with the demonstration of mucosal antibody in the oviduct and gut of mature female chickens infected with the enterotropic IBV strain G. The relationship between antibodies in the serum or oviduct washes and egg production was also investigated.

## 6.2. Materials and Methods

### 6.2.1 Virus

The IBVG strain (El-Houadfi et al., 1986) was titrated in TOC as described in Chapter 3. Each chicken received 200µl of 4.5 $\log_{10}$ $CD_{50}$ of virus in 0.1 ml, oculo-nasally.

### 6.2.2 Chickens

Commercial SPF eggs were hatched in the department and the chicks were reared in strict isolation. Food and water were provided *ad-libidum*.

### 6.2.3 Tracheal organ cultures

TOC were prepared as described in Chapter 3 and used for virus isolations and neutralisation tests.

### 6.2.4. Virus isolations

Virus isolations from tracheal and cloacal swabs were performed in TOC using complete ciliostasis as the criterion for presence of virus. A maximum of three passages was given and the ciliostatic virus was identified by IF staining of unfixed TOC rings (Chapter 3, Bhattacharjee et al., 1994).

### 6.2.5. Immunofluoresence

Samples of selected tissues were snap-frozen in liquid nitrogen and cryostat sections were used for IBV-specific antigen detection by IF staining using hyperimmune rabbit antiserum to M41 strain (kindly provided by Dr. Cook, Intervet, Houghton, UK) and a commercial fluorescein isothiocyanate-labelled anti-rabbit IgG (Sigma). The procedure is described in Chapter 3.

### 6.2.6 Virus neutralisation test

Equal volume pools of serum, tracheal or oviduct washes from infected birds were examined for VN antibodies to strain IBVG (100 $CD_{50}$) in TOC as described in Chapter 3.

### 6.2.7 Haemagglutination inhibition test

Phospholipase-treated, IBV G antigen was used to detect HI antibodies in washes and contents following the method described in Chapter 3.

### 6.2.8 Enzyme linked immunosorbent assay

The indirect ELISA was essentially that described by Mockett and Cook (1986). Purified IBVG (Chapter 3) was used as antigen at a dilution of 1:50 in 50 mM carbonate buffer pH 9.6. Virus-coated plates were incubated at $4^0$C overnight. The wells were blocked with PBS containing 3.0% bovine serum albumin (BSA). In Experiment 1 (Section 6.2.9.1.2, see below), the samples of washings and contents were tested at a single dilution of 1:10 in PBS containing 0.05% tween-20 (PBST) and 1% BSA (dilution buffer). Mouse Mab against chicken IgG and IgA (Mockett,1986) were used at a dilution of 1: 100, followed by affinity-purified goat anti-mouse IgG-peroxidase conjugate (Sigma) at a dilution of 1:500.

In the second and third experiments (Sections 6.2.9.2. and 6.2.9.3.), serial dilutions of sera and tracheal or oviduct washes were made in dilution buffer and tested using affinity-purified rabbit anti-chicken IgG (whole molecule) peroxidase conjugate (Sigma) at a dilution of 1:1000, for the estimation of total IBV-specific IgG antibodies. The baseline values for these samples were established from the mean plus twice the standard deviation of the absorbance values of dilutions of 20 normal samples collected from 8-9 -week old SPF chickens. Titres ($log_2$ units) of antibody in these samples were determined graphically (Mockett and Darbyshire, 1981).

In both ELISAs, the substrate used was O-phenylene diamine (OPD) (0.4 mg per ml in phosphate-citrate buffer, see Appendix). The reaction was stopped by adding 25µl of 2.5 N sulphuric acid and the plates read at 490nm in a micro-ELISA reader (Dynatech). Volumes of 50µl were used throughout except for the blocking step where 100µl was used. The plates were washed three times after each step except after the blocking step.

### 6.2.9 Experimental Designs

#### 6.2.9.1. Experiment 1: Hens infected at 16-weeks of age

##### 6.2.9.1.1 Virus isolation

Six 16-week old female chickens were inoculated with 200 $\mu$l of IBVG (4.5 $\log_{10}$ $CD_{50}$) by the oculo-nasal route. Cloacal and tracheal swabs were collected on days 5, 7, 12, 17 and 21 p.i. for virus isolation in TOC. Cryostat sections of trachea, Harderian gland, duodenum, caecal tonsils and oviduct collected on days 7, 17 and 23 p.i. were used for IBV-specific antigen detection using IF tests.

##### 6.2.9.1.2. Immunoglobulins in washes and tissue contents

On days 7, 17 and 23 p.i., two infected birds and one uninfected control were bled and tears were collected. Post mortem, tracheal washes, duodenal contents, caecal contents, oviduct washes, and bile were also collected. The collecting medium used was phosphate buffered saline (PBS, pH 7.2) except for caecal contents for which PBS containing 50mM EDTA, 0.1 mg per ml soybean trypsin inhibitor (Sigma) and phenyl methyl suphonyl fluoride (Sigma) in absolute ethanol (final concentration 2.0 mM) was used.

Tears were induced by applying approximately 5 mg of sodium chloride crystals to each eye and collecting the lachrymal fluid by micropipette (Toro et al, 1993). Three ml volumes of PBS was injected into 8 cm length of trachea and oviduct and both ends were sealed with bulldog clips. After a gentle massage, the washes were collected by allowing it to flow down by gravity into an universal. All the contents and washings were tested for antigen-specific and/or class-specific (IgG and IgA) antibodies against IBV using ELISA, VN and HI tests. The OD values of washes and contents from infected hens were compared with the mean OD of control birds for significant differences using student's t-test ($p<0.05$).

The assessment of local antibody secretion in certain tissues was performed as per the description of Zigtermann et al. (1993). Selected tissues (Harderian gland, spleen, duodenum, caecal tonsil and magnum, the albumen-secreting region, of the oviduct) were removed and washed thoroughly in Eagles MEM containing 250 IU per ml penicillin and 250 $\mu$g per ml of streptomycin. Three sq.mm pieces were made and three pieces put into a single tube with 1 ml medium. Immediately, 200 $\mu$l of medium were collected from each tube (t = 0). Duplicate sets of tissue were incubated at $37^0$C and $4^0$C. After 18 hours (t=18), the medium was collected from three replicate tubes per bird and assayed for IBV-specific and isotype-specific (IgG and IgA) antibodies using

ELISA. The increase in OD was calculated by comparison with t=0 values. A significant reduction in OD at $4^0$C compared to 37 $^0$C was deemed to be indicative of local active secretion of antibodies. Levels of significance were calculated using student's t-test ($p<0.05$).

#### 6.2.9.2. Experiment 2: Transudation of circulating antibody

In order to determine whether antibodies transude from the serum into the oviduct, three 30-week old SPF hens in-lay weighing 2.1, 2.0 and 1.8 kg were inoculated i.v. with 10.0, 9.0 and 8.0 ml respectively of hyperimmune serum against IBV strain G (with an ELISA titre of $\log_2$ 10.8, Chapter 3). After 40 hours, blood was taken and the birds were killed. Oviduct and tracheal washes were collected and $\log_2$ ELISA titres determined as described by Mockett and Darbyshire (1981).

#### 6.2.9.3. Experiment 3: Comparison of egg production with HI and ELISA titres

Ten SPF hens previously infected with IBVG at day-old and four uninfected controls were monitored for egg production from start of lay to 31 weeks when they were sacrificed. Serum and oviduct washes were collected and assayed for IBV antibodies using ELISA and HI tests and for each individual hen, the titres were compared with egg production.

## 6.3. RESULTS

### 6.3.1. Virus isolation from hens infected at 16-weeks of age

No virus was isolated from tracheal swabs on days 5, 7, 12, 17 and 21 p.i. but it was recovered from cloacal swabs on all three occasions (Table 6.1). By IF staining, viral antigen was found in the epithelium of the oviduct on days 7, 17 and 23 p.i. and Harderian gland on days 7 and 17.p.i. Enteric tissues showed high background staining and the presence of virus could not be confirmed. No antigen was detected in tracheal sections.

**Table 6.1. Isolation of IBV from cloacal and tracheal swabs of infected hens.**

| Bird No. | Days post infection | | | | |
|---|---|---|---|---|---|
| | 5 | 7 | 12 | 17 | 21 |
| 201 | - | -* | | | |
| 203 | - | C* | | | |
| 185 | - | - | - | -* | |
| 258 | - | - | - | C* | |
| 267 | - | - | - | C | C** |
| Untagged | - | - | - | C | C** |

C: cloacal swab positive; -: virus not isolated both from tracheal and cloacal swabs;
*: bird sacrificed. **: bird sacrificed on day 23 p.i.

### 6.3.2. Immunoglobulins in washes and tissue contents

#### 6.3.2.1. IgG antibody in washes and tissue contents

Figure 6.1 depicts the IBV-specific IgG profile in the washings and contents of control and IBV-infected birds. IgG antibody was present in significant amounts in the serum and tears on all three occasions and in oviduct washes on days 7 and 23 p.i. No significant levels of this antibody were detected in tracheal washes nor in the caecal contents on any occasion, but on day 17 p.i. there was a significant concentration of IgG antibody in the duodenal contents.

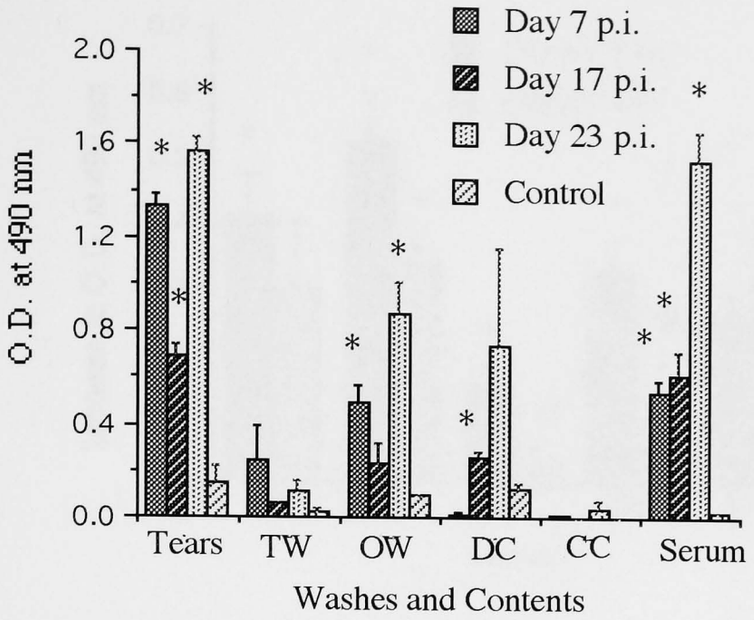


**Figure 6.1.** IgG antibodies in tissue washes and contents after IBV infection. TW: tracheal washes, OW: oviduct washes, DC: duodenal contents, CC: caecal contents. Vertical bars indicate standard deviation. * indicate significantly different from similar samples from control birds.

### 6.3.2.2. *In-vitro* IgG antibody production

Figures 6.2, 6.3 and 6.4 show the results of *in-vitro* IgG antibody production at $37^0$C and $4^0$C on days 7, 17 and 23 p.i. On day 7 p.i., there was a significant reduction in the antibodies detected at $4^0$C in the Harderian gland and oviduct, indicating a possible local synthesis of IgG in these organs.

On day 17 p.i, local synthesis of IgG antibody was still seen in the Harderian gland and in caecal tonsil but no corresponding antibody was detectable in the caecal contents (Figure 6.1). In the oviduct and duodenum, though the ODs were significantly different from the controls (Table 6.2), they were not significantly reduced at $4^0$C.

On day 23 p.i., only the oviduct showed antiviral IgG significantly different from the controls, but there was no reduction at $4^0$C. The ODs of the spleen tissue supernatants incubated at $4^0$C showed an increase (rather than decrease) at $4^0$C on days 7 and 23 p.i., and only a slight decrease on day 17 p.i. The oviduct tissue from day 23 p.i. incubated at $4^0$C also showed an increase in OD.

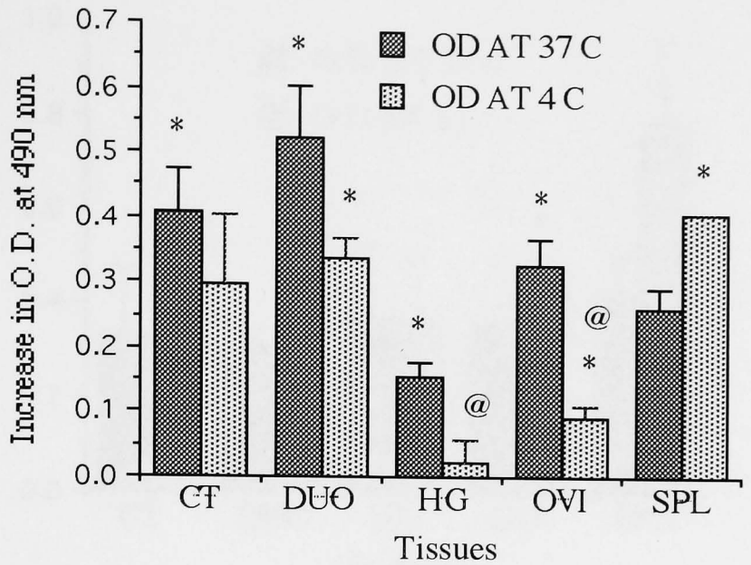


**Figure 6.2.** Local IgG antibody production in supernatants of explants from selected tissues taken from hens at 7 days p.i. CT: caecal tonsils, DUO: duodenum, HG: Harderian gland, OVI: oviduct and SPL: spleen. * indicates significant differences from ODs of supernatants of similar tissues from control hens. @ means significant reduction in OD values of supernatants from explants incubated at $4^0$C compared to $37^0$C.

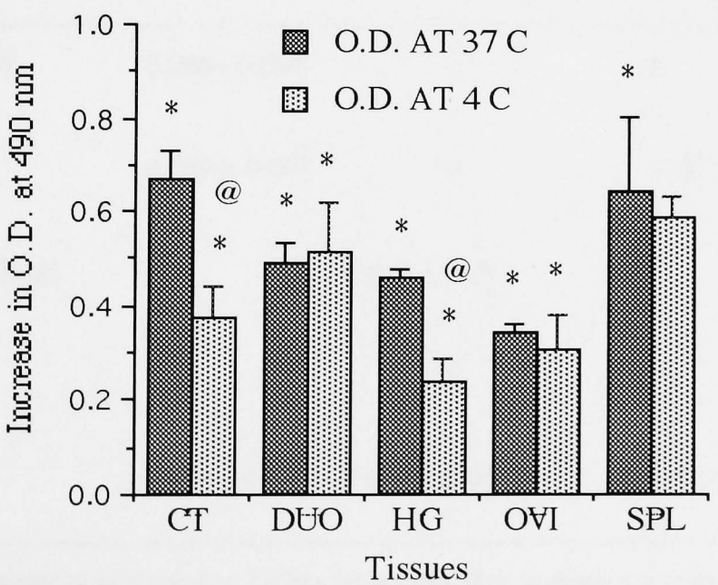


**Figure 6.3.** Local IgG production on day 17 p.i.

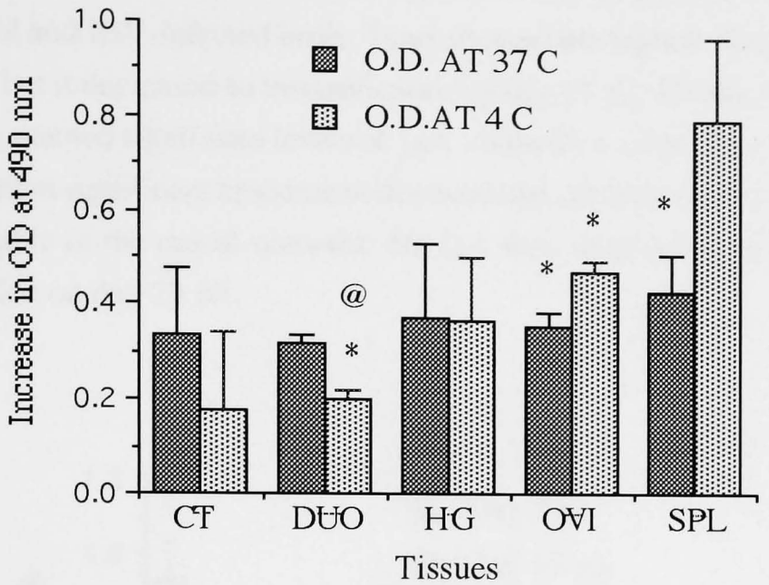


**Figure 6.4.** Local IgG production on day 23 p.i.

**Table 6.2. Mean (± s.d.) increase in OD values in supernatants of explants from selected tissues derived from uninfected control birds, incubated at $37^0$C and $4^0$C**

| Tissues | IgG | | IgA | |
|---|---|---|---|---|
| | Increase in OD at | | Increase in OD at | |
| | **$37^0$C** | **$4^0$C** | **$37^0$C** | **$4^0$C** |
| Caecal tonsil | 0.006± 0.053 | 0* | 0 | 0 |
| Duodenum | 0.269 ± 0.001 | 0 | 0.389 ± 0.012 | 0 |
| Harderian gland | 0 | 0.019 ± 0.011 | 0 | 0.146 ± 0.002 |
| Oviduct | 0 | 0 | 0 | 0.036 ± 0.002 |
| Spleen | 0.254 ± 0.006 | 0.197 ± 0.015 | 0.069 ± 0.011 | 0.060 ± 0.004 |

* : The OD values at time t = 18 hrs less than that at time t = 0 hrs were considered 0.

### 6.3.2.3. IgA antibody in washes and tissue contents

Figure 6.5 shows the anti-viral IgA antibody profile of washings and contents of control and IBV-infected birds. Tears showed the highest level of this antibody on day 7 p.i. but it decreased to insignificance by day 17 p.i. Oviduct and tracheal washes and bile contained significant levels of IgA antibody on day 7 p.i. only. IgA antibody was present in significant amounts in the duodenal contents on day 17 p.i., while none was detecable in the caecal contents. No IgA was seen in any of the contents and washes sampled on day 23 p.i.

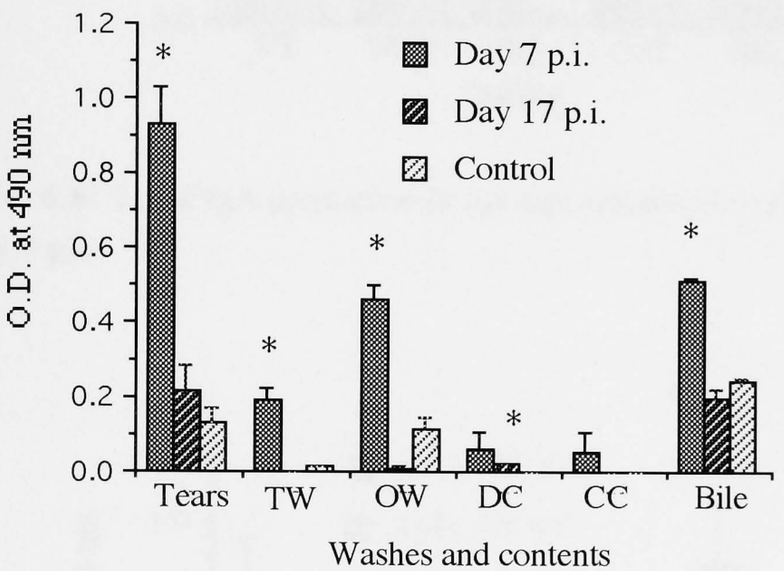


**Figure 6.5.** IgA antibodies in tissue washes and contents after IBV infection. For legend see Figure 6.1.

### 6.3.2.4. *In-vitro* IgA antibody production

Figures 6.6 and 6.7 show the inhibition of virus-specific IgA production at $4^0$C on days 7 and 17 p.i. As expected, there was a significant reduction of IgA antibody production by the Harderian gland at $4^0$C. Although the IgA antibody in the oviduct decreased at $4^0$C, the reduction was not significant. On day 17 p.i., only the Harderian gland tissue seemed to be producing IgA antibody locally even though there was no evidence of significant levels of this antibody in the tears at this time (see figure 6.5).

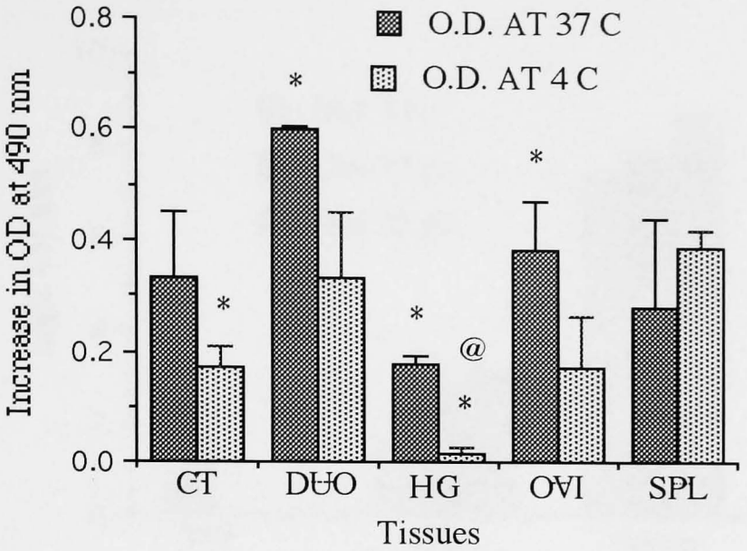


**Figure 6.6.** Local IgA production in the supernatants of explants of selected tissues on day 7 p.i.

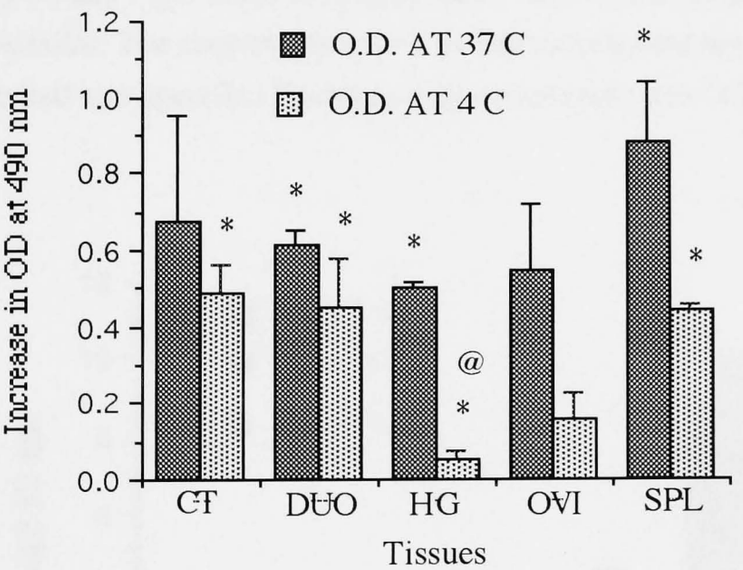


**Figure 6.7.** Local IgA production on day 17 p.i.

Figure 6.8 shows the VN titres of pooled samples of serum, oviduct and tracheal washes. The tracheal wash showed VN activity only on day 7 p.i., while the pattern of VN activity for serum and the oviduct wash was very similar, although titres were higher in the former.

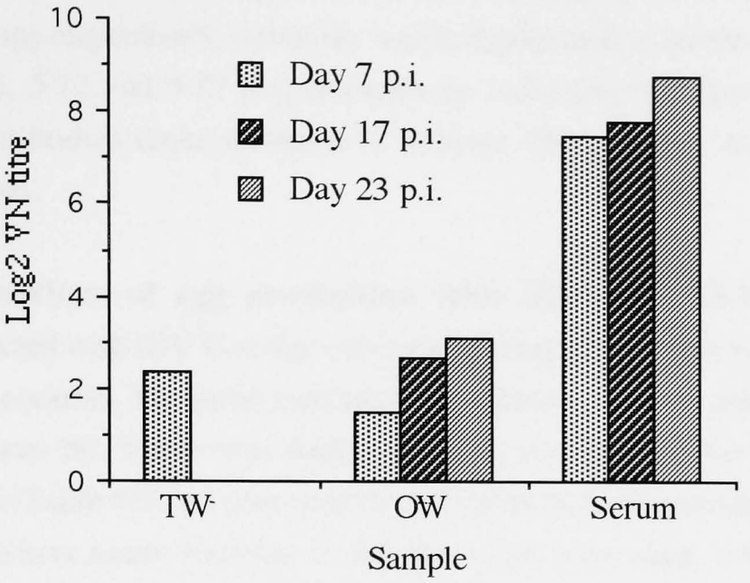


**Figure 6.8.** VN antibodies in pooled tracheal and oviduct washes and serum.

Figure 6.9 shows the HI titres of washings and contents. Tracheal washes showed HI activity only on day 7 p.i. while the pattern of HI activity for serum and oviduct washes were again similar. The control tracheal or oviduct washes did not show any HI activity while serum had non-specific HI activity upto a maximum titre of $\log_2 3.0$.

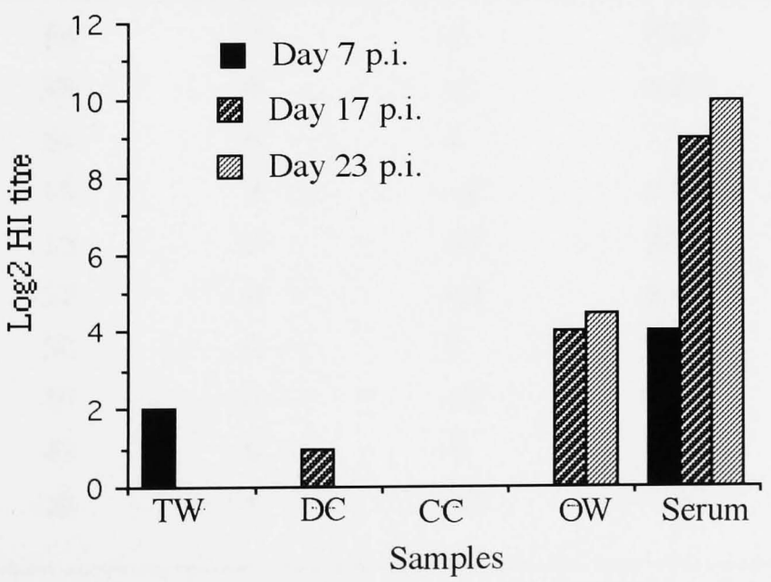


**Figure 6.9.** HI titres in pooled samples of washes and contents.

### 6.3.3. Transudation of circulating antibody

The ELISA antibody titre of the inoculated IBV hyperimmune serum was 10.8 $\log_2$ while those of the sera from recipient chickens at 40 hours post-inoculation were 10.1, 9.7 and 8.9 $\log_2$ respectively. Antibody was demonstrated in all the oviduct washes at levels of 5.72, 5.72 and 4.72 $\log_2$ respectively, indicating that there was transudation of passive antibodies from serum in to oviduct. No antibody was detected in the tracheal washes.

### 6.3.4. Comparison of egg production with IBHI and ELISA

The hens infected with IBV G at day-old came into lay at least two weeks later than the uninoculated controls. The mean number of eggs laid by the four control hens from 20 - 31 weeks was 50. There was wide individual variation in the egg production in infected birds (Table 6.3). At post-mortem, all the birds had apparently normal oviducts except one, where many follicles of the same size were seen, which could indicate delayed passage of the egg. ELISA and HI tests were conducted on the serum and oviduct washes of these birds and their titres correlated with egg production.

**Table 6.3. Comparison of HI and ELISA titres of serum and oviduct washes (OW) with egg production of hens infected with IBVG at day-old.**

| Bird No. | No. of eggs laid[a] | HI titres[b] | | ELISA titres[b] | |
|---|---|---|---|---|---|
| | | Serum | OW | Serum | OW |
| 192 | 54 | 7 | 4 | 8.62 | 5.22 |
| 120 | 38 | 4 | <2 | 6.82 | 5.82 |
| 261 | 51 | 6 | 4 | 7.92 | 4.32 |
| 193 | 13 | 4 | <2 | 6.92 | 6.02 |
| 264 | 15 | 5 | <2 | 8.42 | 5.92 |
| 273 | 27 | 0 | <2 | 4.42 | 4.42 |
| 269 | 52 | 6 | 5 | 8.02 | 4.82 |
| 218 | 19 | 0 | <2 | 4.22 | <2 |
| 208 | 43 | 6 | 4 | 8.32 | <2 |
| 191 | 20 | 4 | <2 | 7.32 | <2 |

a : No. of eggs laid from 20 - 31 weeks of age.; b : $\log_2$ titres

Maximum positive correlation (r=0.889, p=0.001) was seen between the HI titres of oviduct washes and egg production. The birds having higher HI titres in the oviduct washes produced more eggs than those with lesser titres. The coefficient of correlation of serum HI titres with egg production was 0.613, serum ELISA titres 0.467 and oviduct washes ELISA titres 0.125.

## 6.4. DISCUSSION

Though the episodes of virus shedding from birds infected at 16-weeks of age were erratic, IBV was recovered more frequently from the cloacal swabs than trachea as reported earlier with this strain (Jones and Ambali, 1987), on which basis the IBV G strain was proposed as an enterotropic strain. Lucio and Fabricant (1990) have also reported broad tissue distribution of cloacal isolates of IBV, but their tropisms and persistence of infection differed from one another.

Immunoglobulins (Ig) are synthesised in the Harderian gland of the chicken (Albini et al., 1974). Davelaar and Kouwenhoven (1976) have described activation of plasma cells in that gland following IBV infections and have also demonstrated synthesis of IgA by, and its secretion from the Harderian gland following IBV inoculation (Davelaar et al., 1982). In our studies it was seen that after oculo-nasal inoculation of IBV, local production of IgA antibody occurred in the Harderian gland on days 7 and 17 p.i. with corresponding secretion of this antibody in the tears on day 7 p.i. only. Baba et al. (1988) have shown that Ig in the tears originates from the Harderian gland. Local production of IgG antibody was also demonstrated in the Harderian gland of IBV-infected birds on days 7 and 17 p.i., while IgG antibody in the tears was seen on all the three occasions. Though it is generally accepted that IgA is locally produced in the Harderian gland and there is transport of IgG into the tears from serum (Toro et al., 1993), Baba et al. (1990) have shown IgG (about a third to a half of IgA) in *in-vitro* culture supernatants of Harderian gland. Since IgG and IgA ELISAs incorporated different Mabs, the results from the two assays cannot be compared directly (Cook et al., 1992) and they do not necessarily indicate higher levels of antiviral IgG than IgA.

Our finding of anti-viral IgA in the tracheal washes on day 7 p.i. only, correlates with the observations of Hawkes et al. (1983). They also reported that IgG titres in tracheal washes were maximal by day 10 and remained high until at least day 27 p.i. However, in our study we could detect no significant IgG antibody in the tracheal washes. This discrepancy may be due to the relatively shorter time of replication (recovery) of strain IBVG in the trachea (7 days) reported earlier (Jones and Ambali, 1987).

In the oviduct washes, higher levels of IgG antibody on day 7 p.i. could have been either due to local secretion or transudation from the serum, probably favoured by inflammatory changes in the oviduct caused by the virus. The first probability appears more likely as the *in-vitro* antibody production by the oviduct tissue at $4^0$C was significantly reduced compared to that at $37^0$C. This the first ever evidence of local antibody synthesis by the chicken oviduct in response to IBV. However, the *in-vitro* IgG production by chicken oviducts was inhibited at $4^0$C only on day 7 p.i. but not on days 17 and 23 p.i., suggesting that there may be a local synthesis of IgG initially followed by transudation from the serum. The transudation experiment clearly demonstrated passive transfer of IBV antibody into the oviduct.

The IgA produced by the oviduct tissue, decreased compared with that at $37^0$C, but the decrease was not statistically significant, probably because of small number of the birds used. The present study demonstrated that chicken oviducts produce IBV-specific IgA and IgG antibodies locally, in addition to Igs transuded from the serum later in the course of infection. Thus, it may be concluded that, as in mammals, chicken oviduct is also a component of the secretory immune system.

IF studies revealed the presence of viral antigen in the oviduct on all three sampling occasions, together with antibody. When laying hens were infected with the IBV strain M41, viral antigen was detected in oviducts only for 9 days p.i. (Jones and Jordan, 1971). The longer persistence of the IBVG in the oviducts may be related to the virus strain. Thus, whether produced locally or by transudation, the efficacy of antibodies in clearing the virus from the oviduct, remains to be determined.

In the present study, local antibodies were demonstrated in the gut contents of IBV-infected birds. Lutticken et al. (1988) could not demonstrate any antibody in the gut washings of IBV strain M41 vaccinated birds but their sampling time did not start until four weeks after vaccination. It would be interesting to investigate whether the coproantibodies are restricted to the enterotropic strain used here or induced by other strains also.

The assay used in this study for detecting active local secretion of antibodies has been validated to measure mucosal antibody responses in the chicken intestine by Zigtermann et al. (1993). These workers found that inhibition of IgA production at $4^0$C was comparable to that seen following addition of protein synthesis inhibitors (cycloheximide and puromycin) normally used to assess local secretion of antibodies. In our experience, this method was useful except in tissues where there may have been large amounts of extra-cellular antibodies resulting in the OD at $4^0$C being higher than

at 37$^0$C. These extra-cellular antibodies, perhaps not having been removed fully even after repeated washings, would have been preserved better at the lower temperature and produced higher OD values than at 37$^0$C. Interestingly, we found that the ODs of the supernatants of chicken oviducts incubated at 4$^0$C were higher on day 23 p.i., giving indirect evidence of the possible presence of extra-cellular antibodies, resulting from transudation from the serum into the oviducts.

The results of the second experiment clearly indicated that there was transudation of antibody from the serum into the oviducts of uninfected laying birds, in contrast to the trachea, where almost no transudation was seen (Hawkes et al.,1983). However, following IBV infection of chickens, epithelial cell damage in the trachea and consequent inflammatory changes (Purcell and McFerran, 1972) might permit leakage of antibodies from serum into the trachea.

The ELISA and HI tests used on serum and oviduct washes of hens infected with IBV at day-old showed maximum positive correlation between HI titres of oviduct washes and egg production. Box et al. (1988) have shown that the bird's ability to protect against drop in egg production is dependent upon the HI titre of the serum at the time of challenge. Our results indicate that although virtually impracticable to obtain from live birds, the HI titres of oviduct washes may give an indication as to whether an individual hen will lay normally or at a reduced level after a field infection. Further studies are needed with large numbers of birds to confirm this theory.

# CHAPTER 7

## PROTECTOTYPIC DIFFERENTIATION OF AVIAN INFECTIOUS BRONCHITIS VIRUSES USING AN *IN VITRO* CHALLENGE MODEL

**CONTENTS** **PAGE NUMBER**

# CHAPTER 7

## PROTECTOTYPIC DIFFERENTIATION OF AVIAN INFECTIOUS BRONCHITIS VIRUSES USING AN *IN VITRO* CHALLENGE MODEL

### 7.1. INTRODUCTION

IBV poses a constant threat to the poultry industry as new variants of the virus continue to be isolated even from vaccinated flocks (Cook, 1984; Gelb et al., 1991). Antigenic variation among strains of IBV has been analysed by VN or HI tests resulting in a large number of serotypes (Darbyshire et al., 1979; Wadey and Faragher, 1981; Cook, 1984; Brown and Bracewell, 1985; Cook and Huggins, 1986). However, antigenic studies alone do not adequately define immunological relationships between strains since little correlation has been found between serotypes and protection afforded by IB vaccine viruses (Raggi and Lee, 1965). Hence a different approach to IBV typing based on 'protectotypes' was suggested by Lohr (1988). This was intended not only to reduce the large number of serotypes into a smaller number of protectotypes but also to determine whether existing vaccines could protect against newly identified variant IBVs.

Cross immunity tests (CIT) in experimental birds have been performed (Darbyshire, 1980; Darbyshire, 1985; Lambrechts et al., 1993) which have demonstrated varying degrees of cross-immunity among heterologous IBV strains. The use of live birds for reciprocal CIT requires large numbers of individuals and sufficient isolation facilities. To circumvent this problem the use of TOC from IBV immunised birds was suggested (Darbyshire, 1980) and used sucessfully by Hinze et al. (1991), although the differentiation of IBV into tracheal protectotypes, unlike serotypes, was not clear-cut.

IBV has tropisms for epithelial cells of the respiratory tract, kidney, oviduct and gut of chickens. IBV vaccines have always been evaluated on the basis of protection afforded at the level of the trachea, which is a main portal of entry of the virus (McMartin,1993). Protection at the level of the kidneys has also been studied for nephropathogenic IBV (Lambrechts et al.,1993). Protection at the oviduct level has been determined in terms of protection against drop in egg production induced by the challenge virus (Box et al., 1988). No information is available with regard to protection induced by IBV vaccines against direct damage to the oviduct caused by the challenge virus.

Local immunity in the trachea in response to IBV has been extensively studied (Gomez and Raggi, 1974; Gillette, 1981; Hawkes et al., 1983; Nakamura et al., 1991) and it has been shown that TOCs prepared from immunised birds produce a lower titre of the virus than TOC from un-immunised birds, when infected *in-vitro* with virulent IBV (Gomez and Raggi, 1974). It is generally considered that local immunity is important in the trachea and humoral antibodies prevent systemic spread of the virus to other target sites e.g. kidneys and oviduct (MacDonald et al., 1981; Box et al., 1988). However, some virus might still spread to these other organs before the appearance of circulating antibody or escape complete neutralisation. In such circumstances, local antibody in the remote target sites may limit viral replication.

In Chapter 6 it is shown that oviduct washes from IBV-infected hens contain virus-specific IgG and IgA. Although some antibody transudes from the serum, there is evidence of local antibody production in the oviduct tissues. However, the importance of these locally produced antibodies in affording protection to the oviducts against virus-induced damage remains to be investigated.

The aims of the present study were to investigate the importance of local immunity in protection of the oviduct against direct damage and to compare protectotypic classification at the tracheal and oviduct levels, of five IBV strains using an *in vitro* challenge system.

## 7. 2. MATERIALS AND METHODS

### 7.2.1 Chickens

SPF eggs were obtained from a commercial source and hatched in our laboratory. After hatching, different groups of chicks were maintained in flexible isolators (Moredun Isolators, Edinburgh, Scotland) as described in Chapter 3.

### 7.2.2 Viruses

Two commercial IBV vaccines, H120 and D274 and three virulent strains, M41 (Ambali and Jones, 1991a), G (El-Houadfi et al., 1986) and 793/B-like (Chapter 8) were used. All the strains were cultivated in 10-day old embryonated SPF hen's eggs by inoculation via the allantoic cavity. The allantoic fluids were harvested 48 hours p.i and titrated in TOC. Infectivity titres were calculated according to the method of Reed and Muench (1938) and expressed as median ciliostatic doses ($CD_{50}$).

### 7.2.3 Tracheal organ cultures

TOC prepared from 19- or 20-day old embryonated SPF hen's eggs following the method described in Chapter 3, were used for virus titrations. For ciliary scoring, TOC were prepared using tracheas collected from 3-week old normal, vaccinated or infected birds.

### 7.2.4 Induction of precocious oviducts in chicks and OOC

Precocious development of oviducts was induced by oestrogen treatment of unsexed chicks following the method described in Chapter 3. Chickens vaccinated or infected at day-old were given 1 mg of oestradiol benzoate (Intervet, U.K.) subcutaneously on days 11, 15 and 19. Two days after the last injection (three weeks after vaccination or infection) the chickens were killed and oviducts and tracheas collected from females. Males were discarded at post-mortem. OOC were prepared from these precociously induced oviducts as detailed in Chapter 3.

### 7.2.5 Mitogen assays

Proliferation of whole blood cells in response to stimulation with concanavalin A (Con A) and pokeweed mitogen (PWM) was assessed following the method described by Talebi et al. (1995) with modifications. Heparinised blood collected from oestrogen-treated or untreated (normal) chickens at three weeks of age, was diluted 1 in 40 in RPMI 1640 medium with glutamax and 25 mM HEPES (Life Technologies, Irvine, Scotland). Two hundred µl volumes of this diluted blood were added to nine wells of a 96-well flat bottom microtitre plate (Nunc, Denmark). Three wells received 2 µg conA per well in a volume of 20 µl, another set of three wells received 2 µg PWM in a volume of 20 µl while the remaining three wells received the same volume of medium without mitogens. The cultures were incubated at $37.5^0$C in a humidified atmosphere containing 5% $CO_2$ for 56 hours. They were then pulsed with 0.5µCi of $^3$H thymidine (Amersham Life Sciences, Amersham, U.K.) for 16 hours, after which they were harvested using a cell harvester (Skatron Cell Harvester, Herbyen, Norway) and counted in a scintillation counter (Packard Tricarb, Berks, England). The stimulation index (SI) was calculated by dividing the counts per minute (cpm) of mitogen-stimulated cultures by the cpm of unstimulated controls.

### 7.2.6 Experimental Designs

#### 7.2.6.1 Protectotypic and serotypic differentiation of IBV strains

##### 7.2.6.1.1 *In-vivo* vaccination or infection

Eight groups of 12 to 15 chicks were used. Groups 1 to 5 were either vaccinated or infected with H120 and D274 vaccines, M41, G and 793/B-like strains of IBV respectively. Group 6 served as uninoculated controls. The IBV strains were inoculated oculo-nasally into day-old chicks at a dose of 4.5 $\log_{10}$ $CD_{50}$ of virus. On days 11, 15 and 19 oestradiol-benzoate was given to each bird. Three weeks after vaccination or infection, the birds were sacrificed and their tracheas and precociously-induced oviducts were used to make TOC and OOC. These cultures were made from at least five birds in each group. Ten tubes each of TOC and OOC showing 100% ciliary activity were chosen for *in-vitro* challenge.

Groups 7 and 8 were infected with M41 and G strains of IBV respectively but not treated with oestrogen. The neutralisation titres of the serum prepared from these birds were compared with those of similarly infected but oestrogen-treated birds (groups 3 and 4).

##### 7.2.6.1.2 *In-vitro* challenge

Both TOC and OOC prepared from each group of vaccinated or infected birds were challenged *in-vitro* with 3 $\log_{10}$ $CD_{50}$ of the corresponding homologous and the four heterologous viruses. The challenge dose of 3 $\log_{10}$ $CD_{50}$ was chosen based on preliminary studies wherein this dose caused 100% ciliostasis in TOC and OOC derived from uninfected birds by 3 and 5 days respectively.

##### 7.2.6.1.3 Assessment of ciliary activity

After *in-vitro* challenge, TOC were examined for ciliary activity on day 4 post-challenge (p.c.) and OOC on day 6 p.c.

##### 7.2.6.1.4 Ciliary Scoring

Ciliary movement was classified on a scale ranging from 0 (no ciliary movement) to 4 (100% ciliary activity). Thus the maximum score for each set of ten cultures before challenge was 40. After challenge, the cumulative score was assessed for each challenge virus in TOC and OOC.

#### 7.2.1.6.5 Comparison of immunological relationships (protectotypes)

Analysis of the protectotypic relationships among the five IBV strains was performed by the application of the method of Archetti and Horsfall (1950). The cumulative tracheal or oviduct ciliary scores after challenge were used to determine two ratios, $r_1$ and $r_2$. For any two viruses, the ratio $r_1$ was calculated by dividing the heterologous score obtained with virus B by the homologous score obtained with virus A and the ratio $r_2$ was determined by dividing the heterologous score obtained with virus A by the homologous score obtained with virus B. The geometric mean of these ratios (R) was then used to express the reciprocal relationships as a percentage. An R value of 100% represents no protectotypic differences, hence the homologous ratio is always 100%. Two viruses having an R value greater than 50% are considered to be related (Wadey and Faragher, 1981). Those with values with less than 50% were considered unrelated.

#### 7.2.6.1.6 Comparison of antigenic relationships (serotypes)

The birds inoculated with each of the five strains of IBV were bled at three weeks p.i. The pooled sera from each group was used in cross-neutralisation tests in TOC with 100 $CD_{50}$ of each of the five challenge viruses following the method of Cook (1984). The homologous and heterologous neutralisation titres were then used in the Archetti and Horsfall (1950) equation to classify the five strains of IBV into serotypes, using the same criterion for relatedness.

### 7.2.6.2 Oestrogen treatment and immunity

In order to rule out any adverse effect of exogenous oestrogen treatment on the immune responses, two parameters were considered. Whole blood cells collected from oestrogen-treated and normal birds at three weeks of age were compared for their proliferative capacity in response to Con A and PWM.

The homologous and heterologous serum neutralisation titres were compared from three-week old oestrogen-treated and untreated birds, previously infected with IBV strains M41 or G.

### 7.2.6.3 Growth of challenge viruses in oviduct tissues from immunised birds

1000μm pieces of precociously-induced oviduct tissues derived from H120 and D274 vaccinated birds were challenged *in vitro* with the five strains of IBV. Supernatants of infected tissues were collected on days 1, 2 and 3 p.i. and titrated individually in TOC to

assay the infectious virus content. The mean titres expressed according to Reed and Muench (1938) were compared with those obtained by inoculation of the same dose of virus on oviducts derived from uninfected oestrogen-treated birds. Students 't' test was used at 5% level of significance to record any significant reduction in titres of virus from immunised oviducts compared to normal oviducts.

## 7.3. RESULTS

### 7.3.1 *In-vivo* vaccination or infection

The TOC prepared from all the vaccinated or infected chickens had 100% ciliary activity at three weeks p.i. The OOC prepared from H120 and D274 vaccinated chickens had 100% ciliary beating, while those from chickens infected with IBV strains 793/B-like, M41 and G had no motile cilia. One of the birds infected with strain M41 also had a cyst in the oviduct. Therefore, *in vitro* challenge experiments using OOC could be done only with those prepared from birds given vaccine viruses, i.e. H120 and D274.

### 7.3.2 Assessment of ciliary activity

The results of the cumulative scores (expressed as percentage) in TOC and OOC prepared from H120 and D274-vaccinated birds after challenge with homologous and heterologous strains of IBV are shown in Figures 7.1 and 7.2. TOC from birds vaccinated with H120 vaccine and challenged with the homologous virus gave maximum protection, in terms of highest score at 4 days p.c. The protection in OOC was also highest against homologous challenge, although it was only 53.6% of the protection seen at the level of trachea. Protection afforded by the H120 vaccine to the serotypically similar M41 virus was 75% and 80% of homologous protection at the tracheal and oviduct levels respectively. Varying but low levels of cross-protection were seen in TOC and OOC challenged with heterologous viruses.

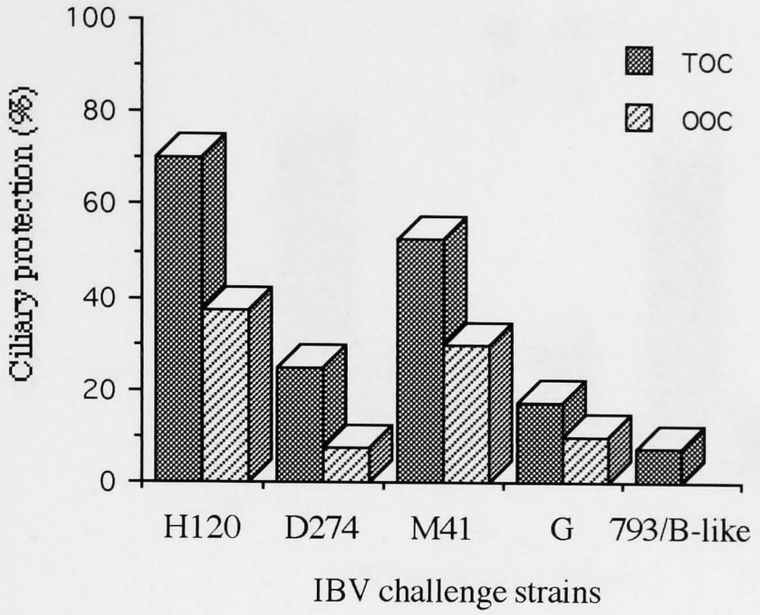


**Figure 7.1** Tracheal and oviduct cumulative ciliary scores of TOC and OOC prepared from chickens vaccinated with H120 vaccine and challenged *in-vitro* with homologous and heterologous viruses.

TOC prepared from birds vaccinated with D274 vaccine gave 93% of homologous protection to a virus belonging to a different serotype (793/B-like). The protection against this heterologous challenge virus, at the level of the oviduct, was only 50% that of homologous protection (Figure 7.2). However, when birds were infected with 793/B-like and challenged with the D274 vaccine virus, protection at the tracheal level was only 42% of homologous protection (Figure 7.3). The chickens infected with strain M41 and challenged with the serologically similar H120 vaccine virus gave 80% of homologous protection to it (Figure 7.4). For strain G infected birds, highest protection was seen against homologous challenge only (Figure 7.5).

All the strains of IBV caused complete ciliostasis in TOC and OOC prepared from unvaccinated birds (group 6) by day 3 and day 5 p.i. respectively.

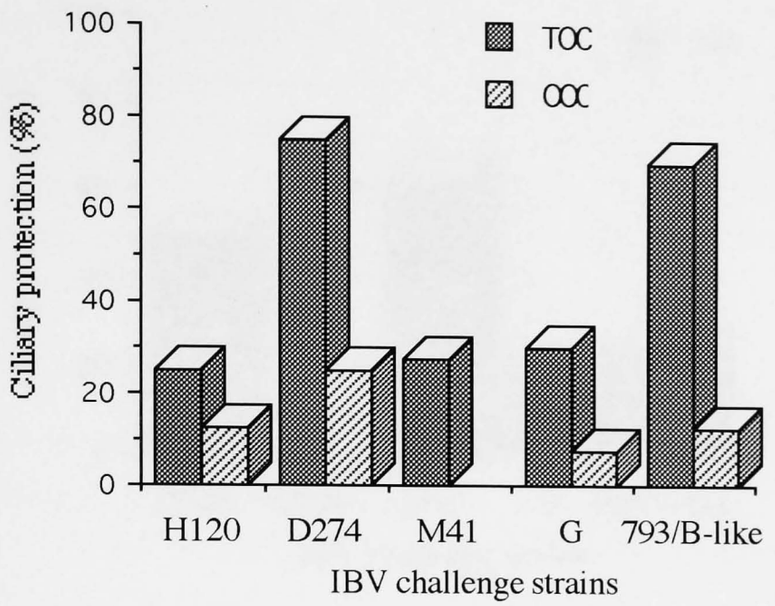


**Figure 7.2.** Tracheal and oviduct cumulative ciliary scores of TOC and OOC prepared from chickens vaccinated with D274 vaccine and challenged *in-vitro* with homologous and heterologous viruses.

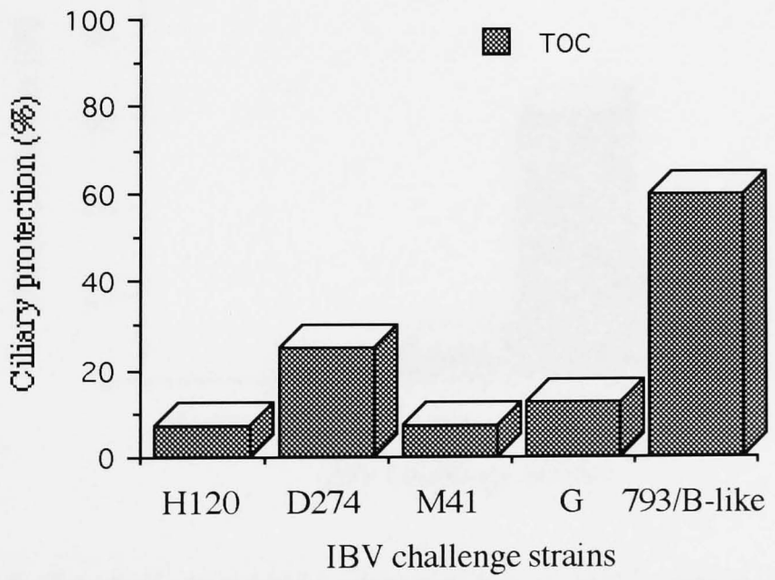


**Figure 7.3.** Tracheal cumulative ciliary scores of TOC prepared from chickens infected with 793/B-like IBV and challenged *in-vitro* with homologous and heterologous viruses.

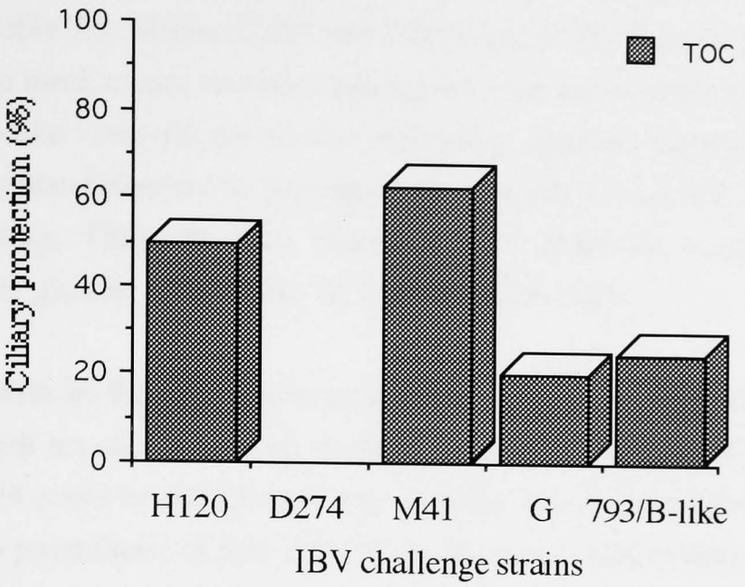


**Figure 7.4.** Tracheal cumulative ciliary scores of TOC prepared from chickens infected with M41 strain of IBV and challenged *in-vitro* with homologous and heterologous viruses.

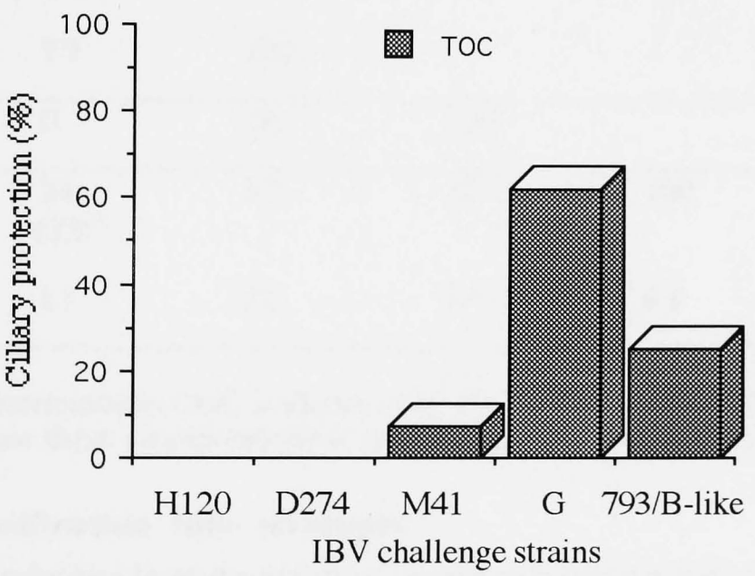


**Figure 7.5.** Tracheal cumulative ciliary scores of TOC prepared from chickens infected with G strain of IBV and challenged *in-vitro* with homologous and heterologous viruses.

### 7.3.3. Classification into protectotypes

The differentiation of the five strains of IBV into tracheal and oviduct protectotypes is shown in Table 7.1. Strains D274 and 793/B had an R value of 62%, which according to the criterion used, meant that they belonged to the same protectotype, although infection with 793/B-like virus did not protect efficiently against challenge with D274. H120 and M41 strains also belonged to the same protectotypic group while strain G belonged to a separate group. Thus, the five strains of IBV could be grouped into three distinct protectotypic groups, M41/H120, D274/793/B-like and G.

Challenge with all the IBV strains except the two vaccines, H120 and D274, resulted in oviducts with no ciliary activity at three weeks p.i. Thus, two-way relationships at the oviduct level could be determined only with the two vaccine viruses and their R value is indicated in parenthesis (Table 7.1). Their degree of relationship at the oviduct level was similar to that at the tracheal level.

**Table 7.1. Protectotypic relationships (R values) of the five strains of IBV in TOC or OOC.**

| IBV strains | H120 | M41 | G | D274 | 793/B-like |
|---|---|---|---|---|---|
| H120 | 100 | | | | |
| M41 | **77** | 100 | | | |
| G | 0 | 20 | 100 | | |
| D274 | 34 (32)* | 12 | 13 | 100 | |
| 793/B-like | 11 | 22 | 29 | **62** | 100 |

* R value determined in OOC is shown in brackets. Values in bold indicate R values more than 50, hence these viruses belong to the same protectotypic group.

### 7.3.4 Classification into serotypes

The R values derived from the results of cross-neutralisation tests in TOC using sera from each group of vaccinated / infected birds are shown in Table 7.2. It was confirmed that these five strains belonged to four serotypes, with H120 and M41 belonging to the same serotype, as expected since the H120 vaccine is the 120th egg passage of a strain of IBV belonging to the Massachusetts serotype.

**Table 7.2. Antigenic relationships of the five strains of IBV.**

| IBV strains | H120 | M41 | G | D274 | 793/B-like |
|---|---|---|---|---|---|
| H120 | 100 | | | | |
| M41 | **65** | 100 | | | |
| G | 16 | 8 | 100 | | |
| D274 | 30 | 15 | 14 | 100 | |
| 793/B-like | 18 | 10 | 14 | 16 | 100 |

### 7.3.5 Oestrogen treatment and immunity

The mitogen responses of oestrogen-treated and untreated birds to Con A and PWM were not significantly different (Table 7.3).

**Table 7.3. SI (mean ± s.d.) to mitogens of oestrogen-treated and normal birds.**

| | SI | |
|---|---|---|
| Mitogens | Normal birds | Oestrogen-treated birds |
| Con A (n = 6) | 39.033 ± 39.417 | 28.1 ± 15.772 |
| PWM (n = 8) | 9.221 ± 11.062 | 17.299 ± 14.709 |

In cross-neutralisation tests, differences between homologous or heterologous neutralisation titres of sera from oestrogen-treated chickens infected with M41 or G strains of IBV were always within 1.0 $\log_2$ of those from similarly infected but untreated chickens (Table 7.4).

**Table 7.4. Homologous and heterologous reciprocal neutralisation titres of infected and oestrogen-treated or normal chickens.**

| Serum | IBV strains H120 | D274 | M41 | G | 793/B-like |
|---|---|---|---|---|---|
| M41 | 45.3 (38)* | <8 (9.5) | 128 (107.6) | 9.5 (9.5) | <8 (<8) |
| G | <8 (<8) | <8 (<8) | <8 (<8) | 53.8 (107.6) | 11.3 (19) |

* The neutralisation titres of sera from oestrogen-treated chickens are shown in parenthesis.

### 7.3.6 Growth of challenge viruses in oviduct tissues from immunised birds

The titres of challenge viruses in the supernatants of OOC derived from non-immunised and immunised chickens are shown in Figure 7.6. Significant reductions in titres of challenge viruses were seen only in the supernatants of OOC derived from immunised chicken and challenged with the homologous virus (as compared to challenge of the same virus in OOC prepared from un-immunised chickens).The reduction in titre of the M41 strain of IBV challenged in oviducts from H120 vaccinated chickens was close to significant levels (P = 0.057).

## 7.4. DISCUSSION

Gomez and Raggi (1974) showed that the yield of IBV from TOC prepared from immunised chickens was significantly less than that prepared from normal chickens. They also found that when TOC from immunised birds were challenged with 5 x $10^{3.3}$ egg-infectious-doses of IBV, ciliary activity was present in 60% of the cultures. This observation was exploited by Hinze et al. (1991) to develop an *in vitro* challenge model to determine protectotypic relationships between IBV strains at the tracheal level. This study reports the extrapolation of this model at the oviduct level. The advantage of this model is that cultures from a single immunised bird can be challenged with a variety of different isolates. This permits rapid and economical evaluation of the potential of IBV strains and vaccines for cross-protection against newly identified variant viruses. A possible disadvantage of this model is that as the tissues are removed from the host, it is likely that

only local immunity is operative under such conditions. However, this feature enabled us to study the importance of local immunity in conferring protection to the oviducts.

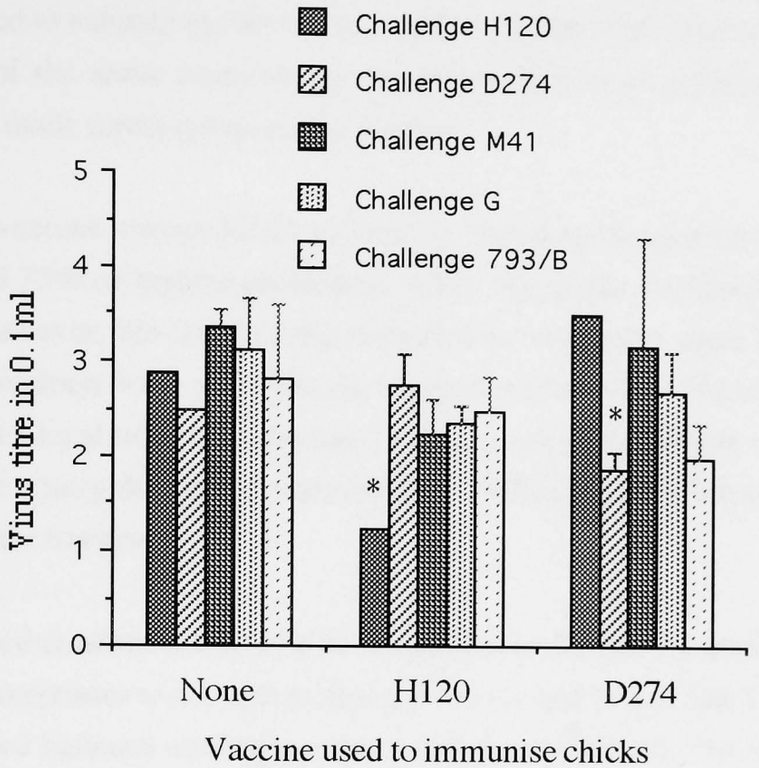


**Figure 7.6.** Mean virus titres from day 1 - 3 p.i., in the supernatants of OOC. Vertical bars indicate standard deviation. * indicates significant differences from titres of un-immunised OOC.

At the level of the trachea, it was seen that serotypically related viruses (H120 and M41 strains of IBV) belonged to the same protectotype, as expected. Surprisingly, two serotypically unrelated viruses (D274 and 793/B-like) appeared to belong to the same protectotype despite a 21% variation in the amino acid sequences of the S1 subunit of the spike protein of these viruses (Adzhar et al., 1995). Protectotypic relationships have also been reported between Australian T and New Zealand A strains of IBV, which are serotypically distinct (Lohr et al., 1991). These findings highlight the potential of protectotypic analysis of IBV strains whereby it may be possible to find an established but unrelated vaccine offering protection to a newly identified variant virus. This could circumvent the need for preparation of a homologous vaccine, which is a long and cumbersome process.

The use of the Archetti and Horsfall (1950) equation with the ciliary score enabled clear differentiation of protectotypes. However, it should be remembered that this formula does not show one-way relationships such as that found between D274 and 793/B-like viruses. The close similarity in relatedness values between H120 and D274 viruses using TOC and OOC appeared to validate the use of the Archetti and Horsfall equation in this context. The application of the same method for the determination of serotypic and protectotypic relationships made direct comparisons possible.

For the two vaccine viruses H120 and D274, homologous protection at the tracheal level was 70% and 75% of highest protection, while that at the oviduct level was only 37.5% and 25%. However, the titres of the homologous challenge virus in the supernatants of immunised oviducts were significantly reduced when compared to the titres in normal oviducts. Thus local immunity appears to play a role in protection of the oviducts against virus induced ciliary damage or replication of challenge virus, but to a much lesser extent compared to the trachea.

To assess whether administration of oestrogen has an influence on cell mediated immunity, proliferative responses to a T-cell mitogen (Con A) and to a B and T-cell mitogen (PWM) were compared between oestrogen-treated and untreated birds. No significant differences were seen in the SIs between the two groups which indicates that oestrogen treatment did not have any adverse effect on T and B-cell responses. However, there was wide variations in the SI values among individual birds. Similar variations have been reported when turkey lymphocytes were treated with oestradiol *in vitro* (Redig et al., 1985). No marked differences were also seen in the neutralisation titres of sera from oestrogen treated and un-treated chickens previously infected with strains M41 and G.

IBV multiplies in the epithelium of the mature oviduct (Jones and Jordan, 1971) and causes drops in egg production and quality. The damage is more severe and permanent if young non-immune chicks are infected (Crinion et al., 1971a). In contrast to oviducts of birds vaccinated with H120 and D274, no ciliary activity was seen in the oviducts of birds infected with M41, G and 793/B-like, when they were examined at three weeks p.i. Hence, although the study of oviduct protectotypic relationships between the three virulent viruses was not possible, this observation may be of value in the evaluation of candidate vaccines for their attenuation and safety at the oviduct level.

Most of the vaccines and vaccination programmes, even for laying and breeding birds, have been evaluated on the resistance to challenge of the respiratory tract rather than of the reproductive system (McMartin, 1993). The use of the respiratory system has been justified on the basis of shorter experiments and because challenge results are easily evaluated. The evaluation of tracheal ciliary activity at four days p.c. is an accepted method for assessing protection and has been found to correlate well with virus isolations and histopathology (Darbyshire, 1980; Andrade et al., 1982; Yachida et al., 1985).

Long-term experiments are needed in order to study protection afforded by vaccines against drops in egg production. Such trials also suffer from lack of reproducibility since the challenge virus may not always cause an uniform drop in egg production (McMartin, 1993). The evaluation of ciliary activity in the oviducts after challenge could be applicable for assessing vaccines in terms of protection against direct damage to the oviduct rather than using long-term egg production experiments. In the present study, the virulent viruses caused stasis of oviduct cilia *in-vivo*, at three weeks p.i. However, for assessing protection of oviducts against *in-vivo* challenge, the earliest time when they cause ciliostasis needs to be determined.

# CHAPTER 8

## IMMUNOPATHOGENESIS OF INFECTION IN SPF CHICKS AND COMMERCIAL BROILERS OF A VARIANT INFECTIOUS BRONCHITIS VIRUS OF ECONOMIC IMPORTANCE

**CONTENTS** **PAGE NUMBER**

# CHAPTER 8

# IMMUNOPATHOGENESIS OF INFECTION IN SPF CHICKS AND COMMERCIAL BROILERS OF A VARIANT INFECTIOUS BRONCHITIS VIRUS OF ECONOMIC IMPORTANCE

## 8.1. INTRODUCTION

Infectious bronchitis continues to be a cause of serious economic problems for the poultry industry and the constant emergence of new serotypes of the virus and their wide and variable tropism for tissues challenge vaccine strategies. A new variant strain of IBV was first reported in the U.K. (Gough et al., 1992; Parsons et al., 1992) and evidence of infection has been shown even in countries like Mexico and Thailand (Cook et al., 1996). The M41-derived vaccine (H120) did not protect chicks against infection with this variant IBV (Parsons et al., 1992). In addition to causing significant mortality in breeders and reduction in egg production, Gough et al. (1992) also reported that this variant (named 793/B) was associated with bilateral myopathy of the deep and superficial pectoral muscles in broiler breeders, a hitherto unreported lesion after IBV infection. However, they could not distinguish it from deep pectoral myopathy of unknown aetiology sometimes observed in breeders (Wight and Siller, 1980). Field evidence has suggested that other features of infection with this variant IBV include enteritis and relatively slow spread and seroconversion (D.B. Pearson, P.W. Cargill and I.R.D. Cameron, personal communications).

The present study describes the immunopathogenesis of infection with an isolate of the variant IBV in day old SPF chicks and 6 week-old broilers. The tissue tropism and infective virus content in the different tissues was examined along with the sites of virus replication. The recruitment of CD4, CD8 and B cells in response to infection was also examined in the trachea, lung and kidneys by immunocytochemistry and a lymphocyte transformation (LT) assay was conducted to see if the virus caused immunosuppression.

## 8.2. MATERIALS AND METHODS

### 8.2.1. Viruses

The virus used in the study (designated 793/B-like) was received as first-passage allantoic fluid from the Ministry of Agriculture Laboratories, Lasswade, Scotland

whence it had been isolated from a recent outbreak of disease. It was purified by four passages in TOC at terminal dilution. It was then passaged twice in 10-day-old embryonated SPF chicken eggs and titrated in TOC as described in Chapter 3, with titres calculated according to the method of Reed and Muench (1938). Confirmation of the identity of this virus was acheived using VN and HI tests. For these tests, the classical Massachusetts M41 virus (Ambali and Jones, 1991a) and the enterotropic variant strain G (El-Houadfi et al., 1986) were also used.

### 8.2.2. SPF chicks and broilers

SPF eggs were obtained from a commercial source and hatched in our laboratory. The chicks were maintained in flexible isolators (Moredun Isolators, Edinburgh, Scotland) as described in Chapter 3. Day-old Ross 1 broiler chicks were maintained in strict isolation pens on litter for 6 weeks at which time they were used. Sera prepared from the broilers when they were three and six weeks old were found to be free of antibodies to the variant IBV by HI and VN tests.

### 8.2.3. Antisera

Antiserum to the reference IBV strain 793/B was obtained from Dr R. E. Gough, Central Veterinary Laboratory, Weybridge. Antisera against IBV strains M41 and G were prepared according to the methods described in Chapter 3.

### 8.2.4. Tracheal organ cultures

These were prepared from 19- or 20-day old SPF chicken embryos following the method detailed in Chapter 3.

### 8.2.5. Monoclonal antibodies

Mab against IgM were used as a marker for B-cells. CD4 and CD8-specific monoclonals were used to identify T-helper and T-supressor/cytotoxic cells respectively. All the Mabs were kindly provided by Dr T. F. Davision of the Institute for Animal Health, Compton, Berkshire.

### 8.2.6. Haemagglutination-inhibition test

The HI test was performed using phospholipase-treated antigen prepared from IBV strains M41, G and 793/B-like, according to the procedure described in Chapter 3.

### 8.2.7. Virus neutralisation test

The VN tests were performed in TOC following the procedure detailed in Chapter 3.

### 8.2.8. Experimental infections:

#### 8.2.8.1. Day-old SPF chicks

Day-old SPF chicks were inoculated oculo-nasally with 4.5 $\log_{10}$ median ciliostatic doses ($CD_{50}$) of 793/B-like virus in a volume of 100$\mu$l. Three infected birds and two uninfected controls were sacrificed on each of days 1, 3, 5, 7, 10, 14 and 21 p.i. Samples of trachea, lung, Harderian gland, oesophagus, proventriculus, duodenum, jejunum, ileum, caecal tonsil, rectum, bursa, kidney and pectoralis muscle were collected individually into pre-weighed vials for virus isolations and titrations. Because of their small size, the Harderian glands were pooled from the three birds at each sampling time. Adjacent pieces of tissue were snap-frozen in OCT compound in liquid nitrogen for IF staining for virus and IP staining for B and T-cells using IgM, CD4 or CD8 markers.

#### 8.2.8.2. Six-week old broilers

These were infected with 4.5 $\log_{10}$ $CD_{50}$ of the virus oculo-nasally in a volume of 100$\mu$l. Three infected and one uninfected control birds were sacrificed on days 3, 7, 10, 14, 21 and 28 p.i. and samples were collected as above. This time, the Harderian gland samples were taken from individual birds.

To test the lateral spread of this virus, five sentinel broilers were placed in contact with the infected birds, three days after inoculation of the latter. Tracheal and cloacal swabs were taken from these birds on days 2, 4, 7, 11, 15, 18 and 21 days post-contact and used for virus isolation in TOC. On day 21 post-contact, the sentinels were bled and their sera tested for HI antibodies.

### 8.2.9. Virus isolations

Virus isolations were performed in TOC using ciliostasis as an indicator of the presence of virus. A minimum of three passages was given and ciliostasis was scored for each passage. The samples which were ciliostatic on the first or second passage were further passaged to establish the specificity of ciliostasis. All the positive samples in the third passage were given another passage and IF staining was done on unfixed infected TOC 24 hours later as described in Chapter 3. Samples positive in the first, second or third passage were given scores of 3, 2, and 1 respectively. Negative samples were scored 0. The scores for the three samples of a given tissue were added to obtain an isolation index (Lucio and Fabricant, 1990). Only those samples which were ciliostatic and positive for IBV antigen by IF staining were used for calculation of the isolation index.

### 8.2.10. Virus titrations

All the samples of like tissues positive for virus isolation on each sampling occasion were pooled and titrated in TOC according to the method described in Chapter 3.

### 8.2.11. Immunofluorescence studies

Adjacent sections of the selected tissues were used in IF tests using antisera to IBVG, for the detection of group-specific IBV antigen following the method detailed in Chapter 3.

### 8.2.12. Histopathology

From broiler birds, sections of trachea, Harderian gland, pectoralis muscle and kidney were examined after fixation of tissues in Bouin's solution and staining with haematoxylin and eosin following conventional histopathology procedures. Only pectoralis muscle was examined from SPF chicks.

### 8.2.13. Immunohistochemistry: CD4, CD8 and B cells

CD4, CD8 and B cells in trachea, lung and kidney were detected in cryostat sections by immunostaining with Mab according to the method of Janse et al. (1994). The numbers of IP-positive cells were quantified using an arbitrary scoring system of +: few cells positive in the field when viewed by a x10 objective, to ++++: 100% of cells positive.

### 8.2.14. Seroconversion

In the broiler experiment, five infected and five controls were bled on days 5, 7, 10, 14, 18, 21 and 28 p.i. On each sampling occasion, sera were tested individually in HI tests and pooled for VN tests.

### 8.2.15. Creatine kinase estimation

In order to assess muscle damage, five identified infected and five control broilers were bled on days 0, 3, 5, 7, 10, 14, 18 and 21 p.i. and their sera used for determination of creatine-kinase (CK) levels using a commercially available kit (Randox Laboratories Ltd., Antrim, UK) following the manufacturer's protocol. The CK levels in units/litre, were expressed as proportional increase over pre-inoculation values for each bird and their mean increase shown (Nyska et al., 1994).

### 8.2.16. Lymphocyte transformation assays

Whole blood collected from five infected and five control broilers on days 0, 5, 11, 15 and 20 p.i. was used for LT assays. Total and differential counts were determined for each sample following the procedure described by Campbell (1988). Blood was diluted in RPMI 1640 medium (Gibco Ltd, Paisley, Scotland) containing 5% foetal calf serum,

to contain 1 x $10^6$ lymphocytes per ml. Fifty $\mu$l of the diluted blood was placed into nine wells of a 96-well flat-bottom microtitre plate. A final concentration of 20$\mu$g per ml Con A and 40$\mu$g per ml phytohaemagglutinin (PHA) were added to triplicate wells. The remaining three wells received only medium and served as unstimulated controls. The cultures were incubated at $37^0$C with 5% $CO_2$ for 72 hours. After 56 hours of incubation, 50 $\mu$l of medium containing 0.5$\mu$Ci $^3$H thymidine was added to each well. The cells were then harvested onto glass filter paper, transferred to scintillation vials and ß-emission counted in a liquid scintillation counter (Packard Co., Pangbourne, Berkshire, England). The results were expressed as cpm after subtracting the cpm of unstimulated controls.

## 8.3. RESULTS

### 8.3.1. Identification of the virus

The presence of IBV in the original allantoic fluid sample was determined by ciliostasis in TOC, dwarfing and curling of chick embryos, coronavirus morphology under electron microscopy and haemagglutination of chicken red blood cells only after treatment with phospholipase C. The untreated allantoic fluid did not show any haemagglutination.

### 8.3.2. Serological comparisons

The results of cross-neutralisation and HI tests using the variant isolate of IBV are presented in Tables 8.1 and 8.2.

**Table 8.1. Cross-neutralisation tests between Massachusetts M41, Moroccan G and the 793/B-like strain.**

| | Virus | | |
|---|---|---|---|
| **Antiserum** | M41 | G | 793/B-like |
| M41 | 1218* | < 8 | < 8 |
| G | < 8 | 1448 | 38 |
| 793/B | 19 | 19 | 431 |

* Figures represents reciprocal neutralisation titres.

**Table 8.2. Serological comparisons of the three viruses by HI test.**

| | Virus | | |
|---|---|---|---|
| **Antiserum** | M41 | G | 793/B-like |
| M41 | 256* | 2 | 3 |
| G | 2 | 192 | 4 |
| 793/B | < 2 | < 2 | 512 |

* Figures represents reciprocal HI titres.

No significant neutraliation or inhibition was seen with M41 or G sera, as there was with the reference 793/B antiserum.

### 8.3.3. Experimental infections

#### 8.3.3.1. Day-old SPF chicks

The day old chicks infected with the variant IBV appeared dull with ruffled feathers until day 3 p.i. Respiratory signs could not be observed clearly since the birds were kept in flexible isolators. No mortality was seen during the 21 day observation period.

Tracheas from chicks killed on days 3 and 5 p.i. were congested and had excess mucus in the lumen and there was congestion of the lungs during this period. The respiratory tissues appeared normal after day 7 p.i. The kidneys were enlarged and pale on day 21 p.i., but urate deposits were not seen. On day 3 p.i. mild pallor of the pectoral muscle was noticed in all three birds sampled. Histopathological examination of the muscle revealed no significant changes, except a patchy edematous separation of muscle fibres on day three p.i. Control birds appeared normal throughout.

#### 8.3.3.2. Six-week old broilers

From day one p.i., the infected broilers showed only mild respiratory signs with coughing and sneezing until day three p.i. Greenish diarrhoea was seen and the litter was always wet till 14 days p.i. At autopsy, tracheas had excess mucus in the lumen on day 3 p.i. but not on day 7. No other gross changes were seen in respiratory tissues. The pectoralis and supracoracoideus muscles were pale and oedematous on day 21 p.i. These muscles could not be neatly incised unlike those of uninfected control birds and the muscle fibres were well separated from each other. Histopathological examination of the tracheas revealed mild changes characteristic of IB such as deciliation and

depletion of mucous glands with infiltration of heterophils and lymphocytes. Accumulations of plasma cells were seen in the Harderian gland connective tissue but there was no other damage. Neither pectoral muscles nor kidneys showed any significant histological changes.

### 8.3.4. Virus distribution in tissues

#### 8.3.4.1. Day-old SPF chicks

***Isolation index:*** The isolation index was highest for oesophagus followed by trachea, kidney, lung, rectum, bursa, caecal tonsil, proventriculus, ileum and duodenum (Table 8.3). The pooled Harderian gland samples showed evidence of virus replication until 7 days p.i. No virus was isolated from jejunum or muscle on any occasion. No virus was isolated from controls.

***Immunofluorescence:*** At various times after infection, viral antigen was detected in trachea, kidney, ileum and rectum. Virus was present in the epithelium of the trachea until 7 days p.i. and in the collecting tubules of kidneys until day 7 p.i. and on day 21 p.i. in SPF chicks. In the ileum and rectum (Fig 8.1), viral antigens were detected in the cytoplasm of the epithelial cells on the tips of villi. Using this technique virus was not demonstrated in other tissues.

**Table 8.3. Isolation index of 793/B-like IBV in young SPF chicks.**

| Tissue | Days p.i. 1 | 3 | 5 | 7 | 10 | 14 | 21 | Index | (order) |
|---|---|---|---|---|---|---|---|---|---|
| trachea | 9 | 9 | 9 | 5 | 0 | 0 | 0 | 32 | (2) |
| lung | 9 | 3 | 3 | 6 | 3 | 0 | 0 | 24 | (4) |
| [Harderian gland[a] | 2 | 3 | 3 | 3 | 0 | 0 | 0 | 11] | |
| oesophagus | 9 | 9 | 8 | 9 | 0 | 0 | 0 | 35 | (1) |
| proventriculus | 3 | 3 | 9 | 0 | 0 | 0 | 0 | 15 | |
| duodenum | 3 | 0 | 3 | 0 | 0 | 0 | 0 | 6 | |
| jejunum | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| ileum | 3 | 0 | 5 | 0 | 0 | 0 | 0 | 8 | |
| caecal tonsil | 6 | 6 | 0 | 3 | 0 | 0 | 0 | 15 | |
| rectum | 3 | 3 | 3 | 6 | 3 | 3 | 0 | 21 | (5) |
| bursa of Fabricius | 4 | 6 | 3 | 3 | 0 | 0 | 0 | 16 | |
| kidney | 3 | 3 | 6 | 6 | 0 | 0 | 9 | 27 | (3) |
| pectoral muscle | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |

a : Harderian glands, because of their small size, were pooled.

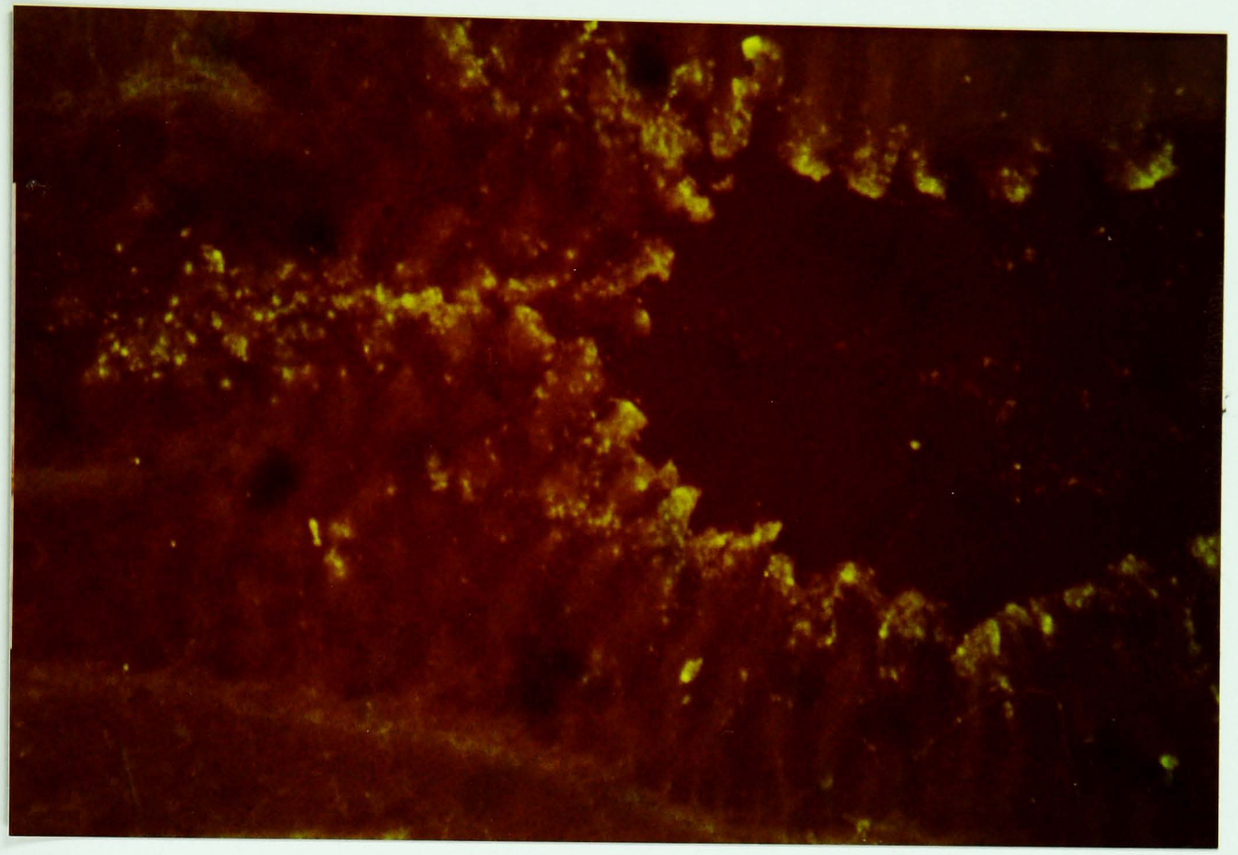

**Figure 8.1** IF staining of a section of rectum of an SPF chick three days after infection. The enterocytes at the tips of villi are strongly stained. Magnification x60.

### 8.3.4.2. Six-week old broilers

***Isolation index:*** The isolation index was highest in ileum, followed by caecal tonsil, rectum, bursa and Harderian gland (Table 8.4). No virus could be isolated from proventriculus, duodenum or muscle on any occasion. No virus was isolated from controls.

***Immunofluorescence:*** Viral antigens were detected in the epithelium of the trachea on day 3 only and in the kidney, ileum and rectum on days 3 and 7. The appearance was similar to that in SPF chicks.

### 8.3.5. Infective virus content of tissues

Table 8.5 shows the titres of virus in the trachea, lung, Harderian gland, bursa and kidney of SPF chicks and broiler chickens. Virus persistence in the trachea and lung was greater in the SPF chicks than in broilers but values for Harderian gland and bursa were similar for both.

For the kidney, virus clearance in both types of bird appeared to be complete by day 10 p.i. However, in the SPF chicks, a titre of 5 $\log_{10}$ $CD_{50}$/g was recorded on day 21, even though the tissue was negative on days 11 and 14.

**Table 8.4. Isolation index of 793/B-like IBV in six-week old broilers.**

| Tissue | Days p.i. 3 | 7 | 10 | 14 | 21 | 28 | Index (order) |
|---|---|---|---|---|---|---|---|
| trachea | 6 | 0 | 0 | 0 | 0 | ND | 6 |
| lung | 6 | 0 | 0 | 0 | 0 | ND | 6 |
| Harderian gland | 8 | 5 | 0 | 0 | 0 | ND | 13 (5) |
| oesophagus | 2 | 0 | 0 | 0 | 0 | ND | 2 |
| proventriculus | 0 | 0 | 0 | 0 | 0 | ND | 0 |
| duodenum | 0 | 0 | 0 | 0 | 0 | ND | 0 |
| jejunum | 9 | 0 | 0 | 0 | 0 | ND | 9 |
| ileum | 9 | 9 | 5 | 0 | 0 | ND | 23 (1) |
| caecal tonsil | 8 | 9 | 0 | 0 | 0 | ND | 17 (2) |
| rectum | 6 | 6 | 3 | 0 | 0 | ND | 15 (3) |
| bursa of Fabricius | 8 | 6 | 0 | 0 | 0 | ND | 14 (4) |
| kidney | 3 | 6 | 0 | 0 | 0 | 0 | 9 |
| pectoral muscle | 0 | 0 | 0 | 0 | 0 | 0 | |

**Table 8.5. Virus titres ($\log_{10}$ $CD_{50}$/g) in respiratory and lymphoid tissues and kidney.**

| Days p.i. | Trachea SPF[b] | Trachea Br[c] | Lung SPF | Lung Br | HG[a] SPF | HG[a] Br | Bursa SPF | Bursa Br | Kidney SPF | Kidney Br |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2.5 | ND[d] | 3.25 | ND | 2.5 | ND | 2.5 | ND | 2.75 | ND |
| 3 | 3.25 | 3.25 | 2.75 | 2.75 | 2.25 | 2.5 | 3.25 | 3.75 | 3.5 | 3.25 |
| 5 | 4.25 | ND | 2.75 | ND | 3.75 | ND | 3.25 | ND | 4.25 | ND |
| 7 | 3.5 | <1 | 3.5 | <1 | 2.5 | 2.5 | 2.25 | 3.25 | 4.5 | 3.75 |
| 10 | <1 | <1 | 2.5 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| 14 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| 21 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | 5.0 | <1 |
| 28 | ND | ND | ND | ND | ND | ND | ND | ND | ND | <1 |

a : Harderian gland; b : day-old SPF chickens; c : six-week old broilers; d : not done

Table 8.6 shows the virus titres in regions of the gut for the two types of bird. For oesophagus, proventriculus, duodenum and rectum, both persistence and titres were higher in SPF chicks than broilers. The reverse was true for ileum. Virus was never detected in the jejunum of SPF chicks but was present at least on day 3 p.i. in broilers. Values for caecal tonsil were approximately similar in both groups. In SPF chicks, virus was still present in the rectum after 14 days p.i., the longest for any tissue examined.

**Table 8.6. Virus titres ($log_{10}$ $CD_{50}$/g) in gut tissues.**

| Day p.i. | Oeso[a] | | Provent[b] | | Duod[c] | | Jejunum | | Ileum | | CT[d] | | Rectum | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SPF | Br | SPF | Br | SPF | Br | SPF | Br | SPF | Br | SPF | Br | SPF | Br |
| 1 | 3.0 | ND | 4.3 | ND | 3.3 | ND | <1 | ND | <1 | ND | 2.5 | ND | 1.5 | ND |
| 3 | 3.5 | 2.3 | 3.5 | <1 | <1 | <1 | <1 | 2.5 | 2.5 | 4.0 | 3.0 | 4.0 | 3.8 | 2.5 |
| 5 | 4.3 | ND | 2.8 | ND | 3.8 | ND | <1 | ND | <1 | ND | <1 | ND | 3.5 | ND |
| 7 | 3.8 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | 2.3 | 2.8 | 3.8 | 2.8 | 2.5 | 3.8 |
| 10 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | 2.3 | <1 | <1 | 2.5 | 2.5 |
| 14 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | 2.3 | <1 |
| 21 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |

a : oesophagus; b : proventriculus; c : duodenum; d : caecal tonsil

### 8.3.6. Immunocytochemistry: CD4, CD8 and B cells

Table 8.7 shows the results of immunostaining of trachea, lung and kidney sections of infected SPF chicks with anti-CD4, CD8 or IgM Mabs. In the trachea, CD8 cells were recruited in response to infection from day 1 p.i. and were at a maximum by day 7 p.i. CD4 cells were not recruited until day 5 p.i. A similar pattern of staining was seen in the lungs. In both cases, CD8 cells outnumbered CD4 cells at the time of virus clearance, viz. 7 and 10 days respectively. In the kidneys, CD8 cells were seen from day 3 p.i. only and their infiltration was less compared to trachea or lungs. CD4 cells were seen only from day 7 p.i. Figures 8.2, 8.3, 8.4 and 8.5 show CD4 and CD8 cells in trachea and kidney at different times p.i. (see legends).

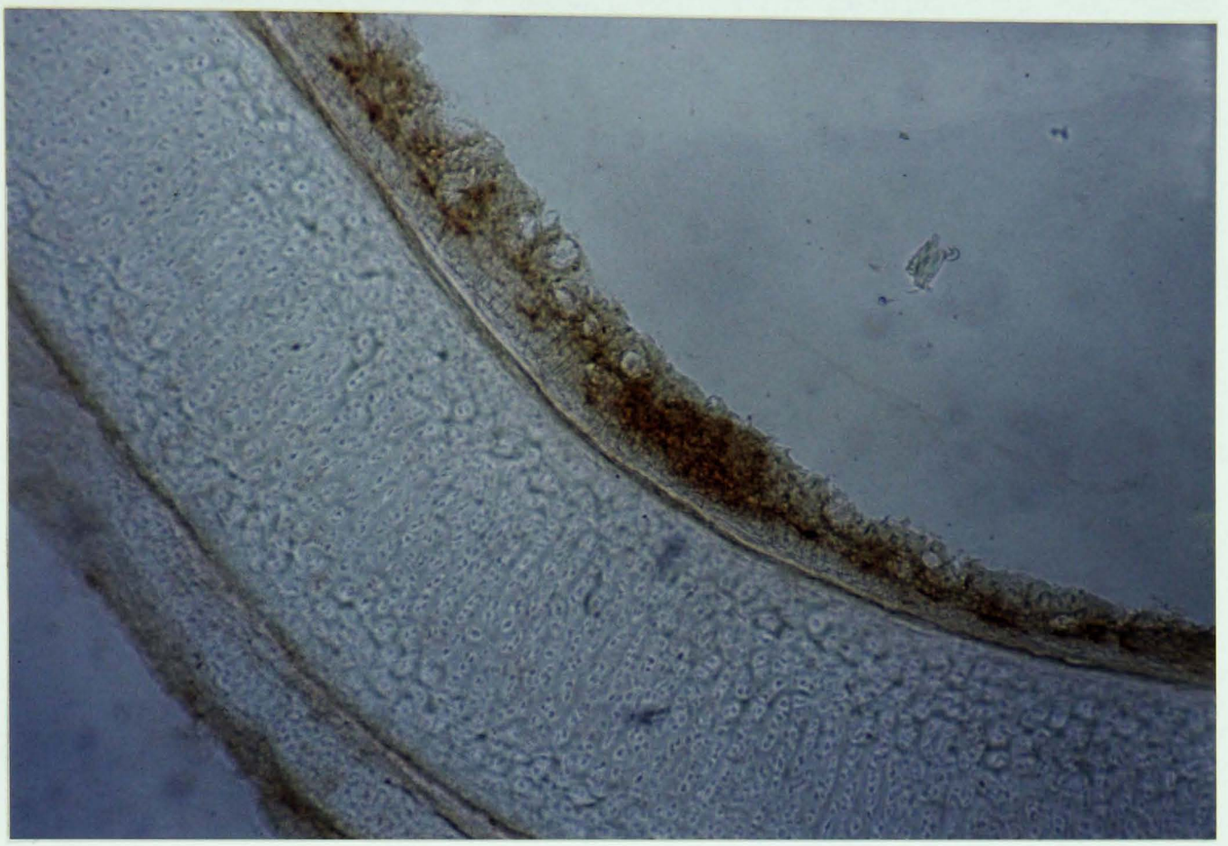

**Figure 8.2.** Trachea of an SPF chick at 7 days p.i. showing T-cells positive for CD4 marker. Magnification x125.

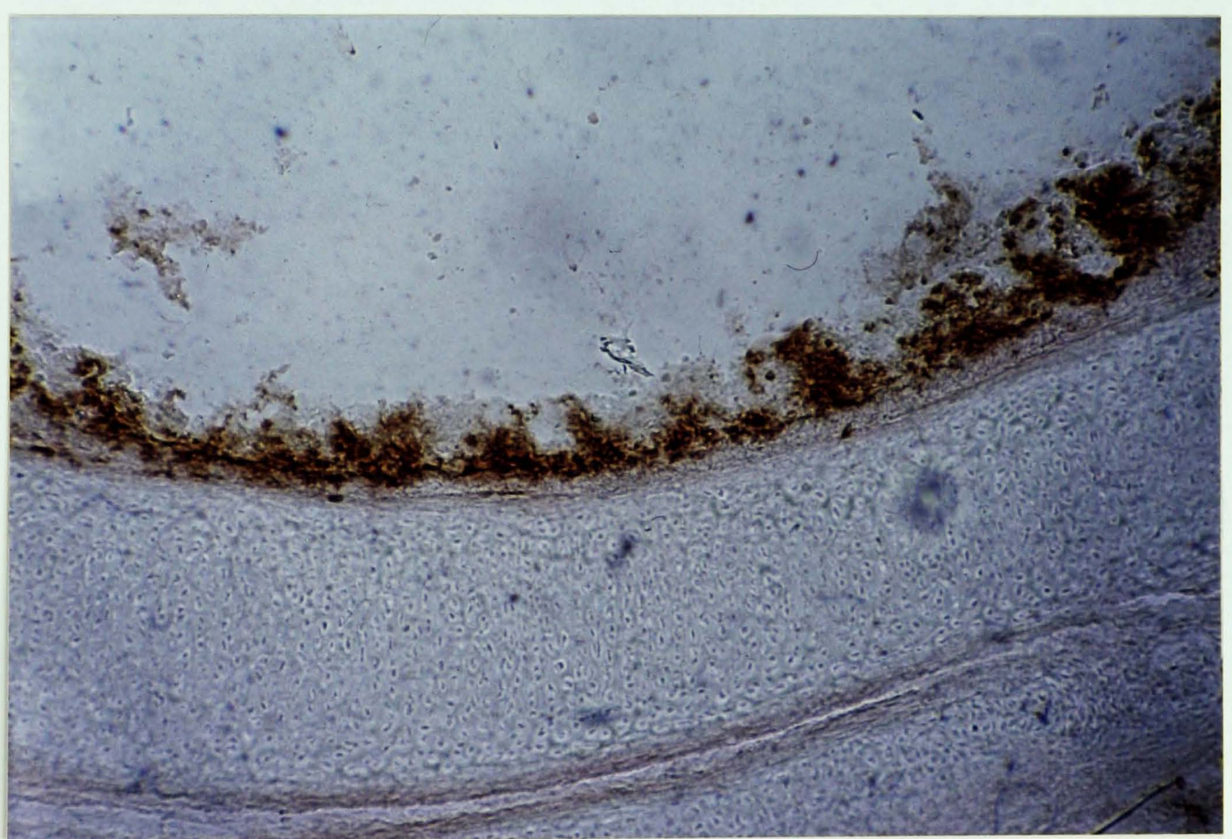

**Figure 8.3.** Trachea of an SPF chick at 7 days p.i. showing heavy staining of T-cells positive for CD8 marker. Magnification x125.

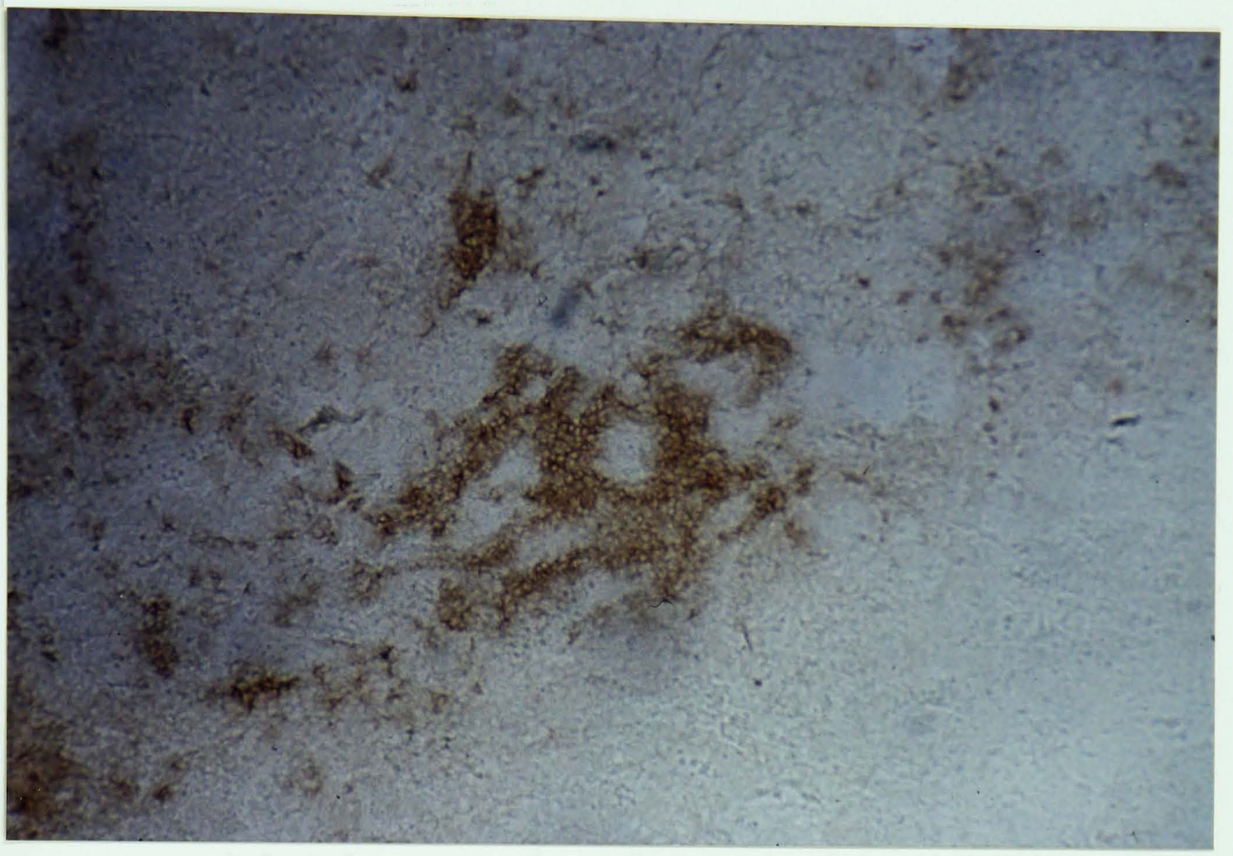

**Figure 8.4** Kidney of an SPF chick at 10 days p.i. showing T-cells positive for CD4 marker. Magnification x125.

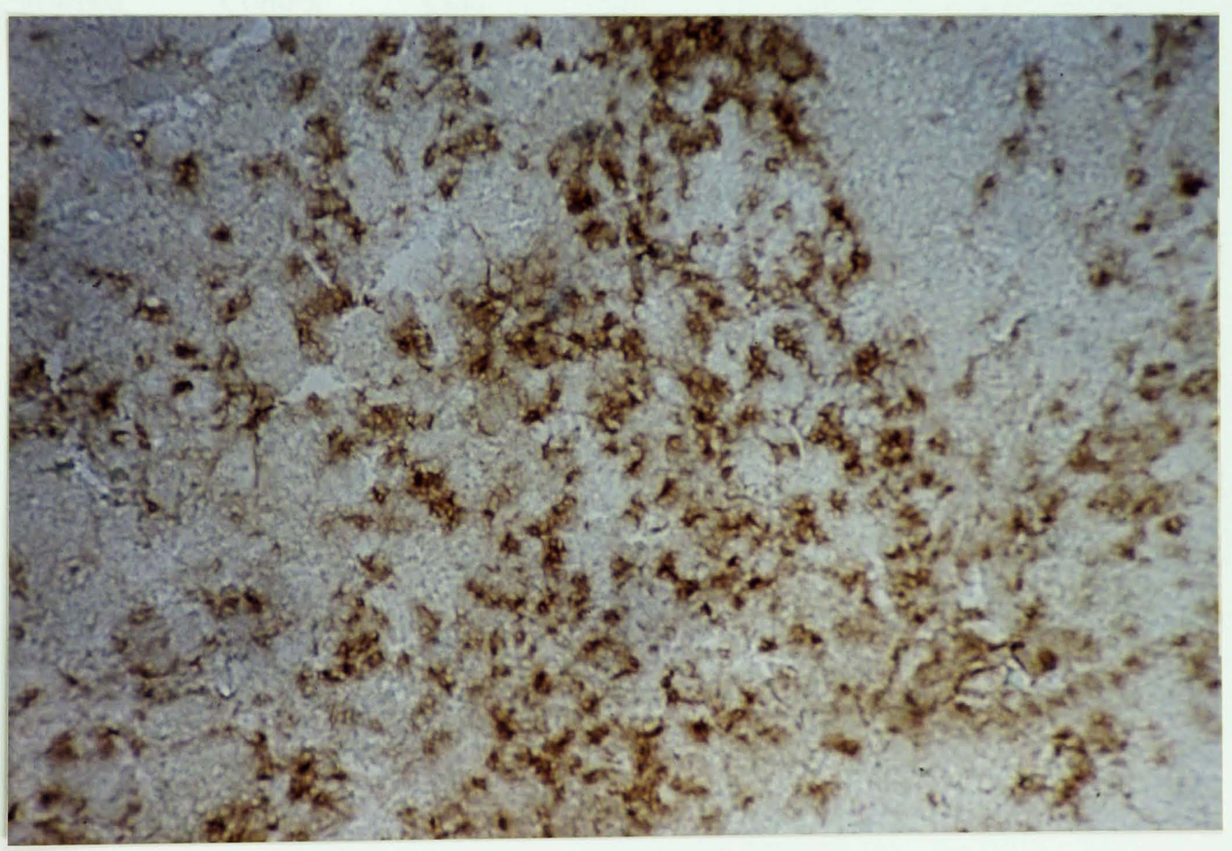

**Figure 8.5** Kidney of an SPF chick at 10 days p.i. showing T-cells positive for CD8 marker. Magnification x125.

In all tissues, B cells were recruited later than T cells (Table 8.7). In the kidneys on days 10 and 14 p.i., IgM antibodies were seen in the mesangium of the glomeruli (Fig.8.6). Virus could not be recovered from these kidneys at this time, although the tissue was positive on day 21. This staining could indicate the formation of soluble immune complexes deposited in the glomeruli. No such staining was seen in the control birds.

Similar patterns of immunostaining for CD8 and CD4 cells were seen in tissues of the broilers. IgM antibodies were detected in the glomeruli of the kidneys as early as 7 p.i. and throughout until day 28 p.i.

**Table 8.7. IP staining of trachea, lung and kidney sections of SPF chickens for T and B-cells.**

| Tissue | Control/ infected | Mab | Days p.i. 1 | 3 | 5 | 7 | 10 | 14 | 21 |
|---|---|---|---|---|---|---|---|---|---|
| Trachea | Control | CD4 | - | - | - | - | - | + | + |
| | | CD8 | - | - | - | - | - | - | + |
| | | IgM | - | - | - | - | - | + | + |
| | Infected | CD4 | - | - | + | ++ | ++ | ++ | +++ |
| | | CD8 | + | + | ++ | +++ | ++ | ++ | +++ |
| | | IgM | - | - | - | + | + | + | - |
| Lung | Control | CD4 | - | - | - | + | + | + | - |
| | | CD8 | - | - | - | + | + | + | - |
| | | IgM | - | - | - | - | - | + | + |
| | Infected | CD4 | - | - | + | + | ++ | +++ | + |
| | | CD8 | + | + | + | ++ | +++ | +++ | ++ |
| | | IgM | - | - | - | + | + | ++ | + |
| Kidney | Control | CD4 | - | - | - | - | - | + | +++ |
| | | CD8 | - | - | - | + | + | + | ++ |
| | | IgM | - | - | - | - | + | + | + |
| | Infected | CD4 | - | - | - | + | + | ++ | ++ |
| | | CD8 | - | + | + | + | + | ++ | ++ |
| | | IgM | - | - | - | + | ++ | ++ | + |

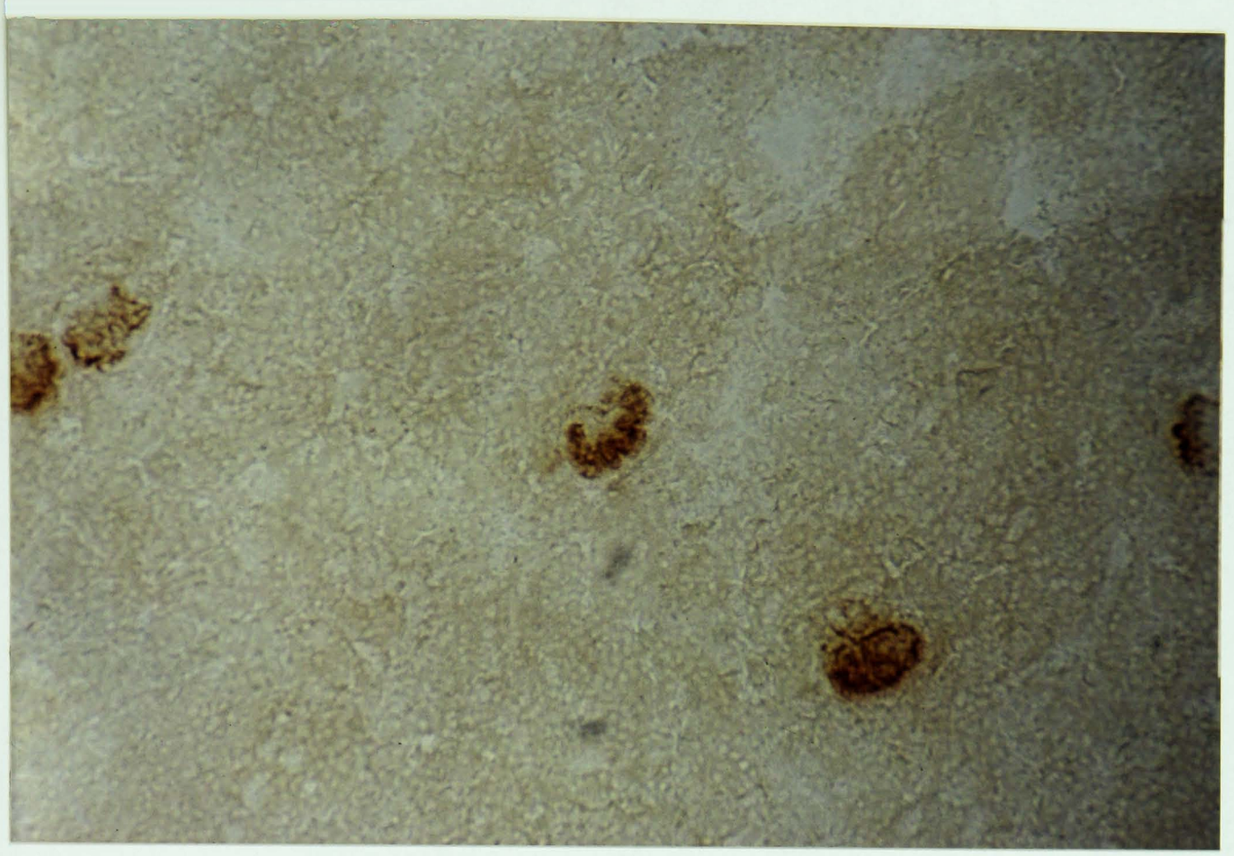

**Figure 8.6.** Kidney of an SPF chick at 10 days p.i. showing cells in the mesangium of the glomeruli which are stained for IgM. Magnification x125

### 8.3.7. Seroconversion

HI antibodies were detected in the sera of infected broilers by day 5 p.i. and reached a peak titre of $\log_2$ 8.0 by day 10 p.i.(Figure 8.7). Serum neutralising (SN) antibodies were present from day 14 p.i.

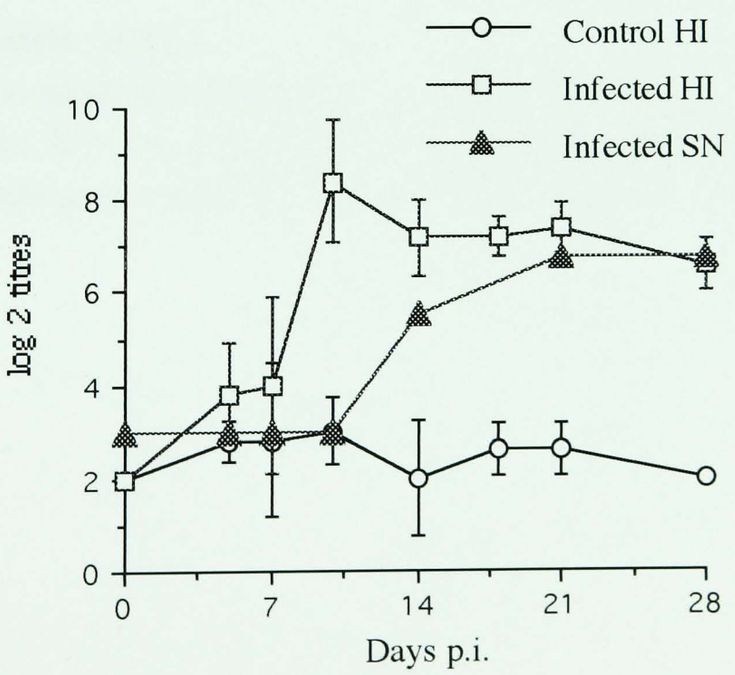


**Figure 8.7** HI and SN titres in the sera of infected broilers

### 8.3.8. Spread of the virus

By day 2 post-contact, four of five sentinels were shedding virus either via the cloaca or trachea (Table 8.8). Shedding persisted intermittently until seven days post-contact, while one bird was positive on day 15 post-contact also. By day 21 post contact, the mean HI titre for the sentinels was $\log_2$ 6.8, compared to the mean of $\log_2$ 7.4 for the inoculated birds at 21 days p.i.

**Table 8.8. Virus excretion by sentinel birds kept in contact with 793/B-like IBV-infected broiler chickens.**

| Bird No. | Days post-contact | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2 | 4 | 7 | 11 | 15 | 18 | 21 |
| 126 | - | C | - | - | - | - | - |
| 127 | C | TC | C | - | C | - | - |
| 129 | T | T | C | - | - | - | - |
| 133 | C | - | - | - | - | - | - |
| 135 | C | TC | C | - | - | - | - |

T: tracheal swab positive; C: cloacal swab positive.

### 8.3.9. Creatine kinase

There were no significant differences in CK levels between controls and infected birds on any of the days the birds were sampled (students t-test; $p<0.05$) (Figure 8.8), although slight gross changes were seen in the pectoral muscles of infected birds on day 21 p.i.

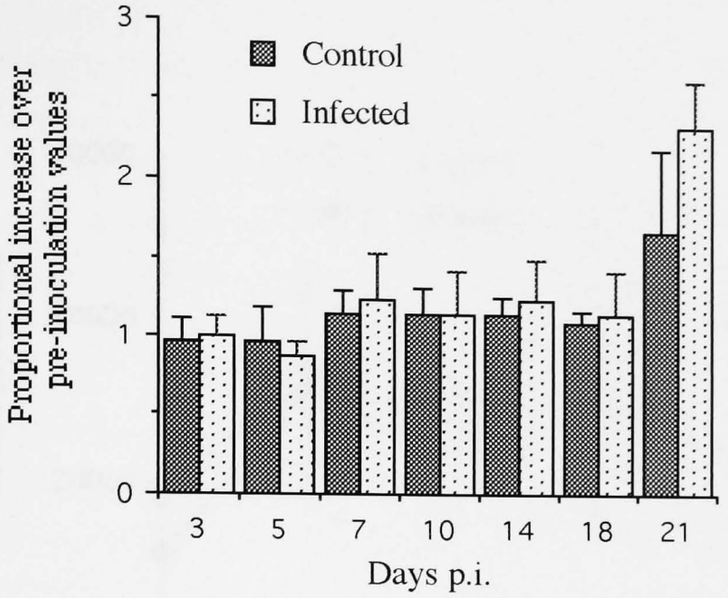


**Figure 8.8** Levels of serum creatine kinase in control and infected broilers.

### 8.3.10. Lymphocyte transformation

Figures 8.9 and 8.10 show the LT responses to mitogens Con A and PHA. In response to Con A the cells from infected birds showed significantly increased stimulation on days 5, 15 and 20 days p.i. (t-test; $p < 0.05$) but not on day 11 p.i.

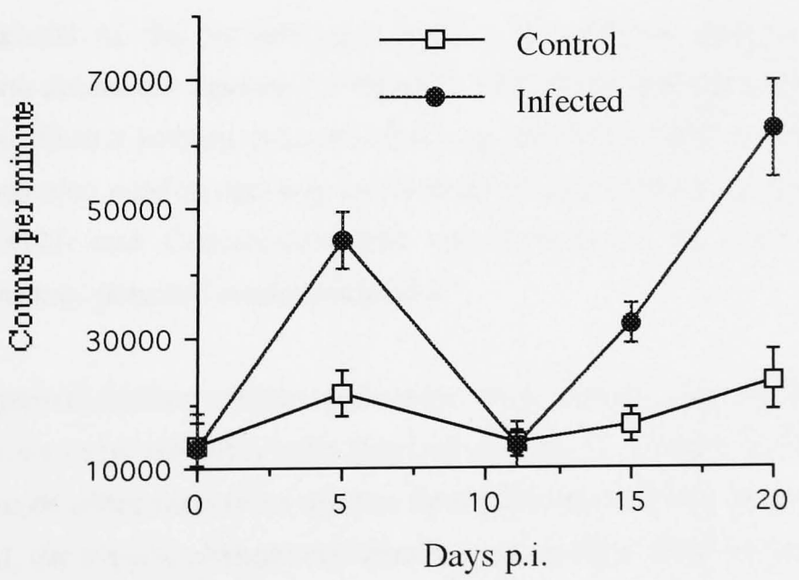


**Figure 8.9** LT responses of control and infected broilers to Con A. Vertical bars represent standard deviation.

No significant differences between control and infected birds were seen in response to PHA on any of the days sampled.

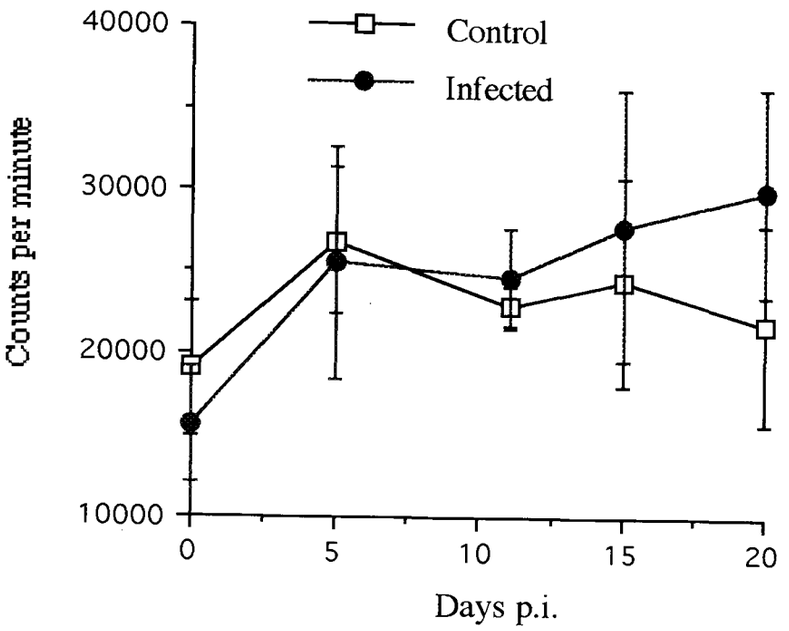


**Figure 8.10** LT responses of control and infected broilers to PHA. Vertical bars represent standard deviation.

## 8.4. DISCUSSION

The identity of the variant virus used in the present study was confirmed using reference antiserum against 793/B IBV. This serum has been shown not to react with M41 and Dutch variant strains D274 and D1466 of IBV (Gough et al., 1992). The virus was also used in one-way cross-neutralisation tests with sera against IB vaccines H52, H120 and Connecticut and was found not to react significantly (K.J. Worthington, personal communication).

Two types of chicken and two ages were used. Firstly, day old SPF chicks were used because we expected them to be most susceptible to infection at this age and they were also free of other infectious agents. Secondly, we used six-week old broilers since in the field, the muscle changes are usually seen in older birds of heavy breeds but not in younger birds (I.R.D. Cameron, personal communication). In practice, marked differences in clinical disease and virus persistence were seen between the two groups. For example, virus was recovered from the respiratory tissues for 7 to 10 days in SPF chicks but for only 3 days in broilers. Thus the virus was cleared more rapidly from

this system in broilers. This feature may have been due to differences in age, breed or housing patterns of the SPF and broiler birds.

The tissue tropisms of IBV strains seem to be wide and varied (Lucio and Fabricant, 1990). The presence of IBV in the respiratory and urogenital tract of chickens is well documented. An enterotropic IBV, strain G, has been shown to have a greater prediliction for the enteric tract as compared to trachea (Jones and Ambali, 1987). Different strains of IBV have been isolated from spleen, faeces, caecal tonsil, cloacal contents, semen, eggs, bursa and oesophagus (Lucio and Fabricant, 1990).

The isolation index of the virus in SPF chicks was highest in the oesophagus. Virus multiplication could not be confirmed in this tissue using indirect IF because of high background staining of control tissues. It is not clear whether the virus detected actually multiplies in this tissue or represents that coughed out from the trachea. An isolate of IBV characterised by Lucio and Fabricant (1990) also had a maximum isolation index from the oesophagus. In the broilers, however, the 793/B-like virus was recovered from the oesophagus only once.

Though virus could be recovered from proventriculus and duodenum of SPF chicks and jejunum of broilers it was also not confirmed whether virus replicated in these tissues. In contrast to the regions of the upper gut, virus multiplication could be conclusively shown in the tips of the villi in ileum and rectum. The more consistent recovery of virus from the rectum up to 14 days p.i. in SPF chicks and 10 days in broilers emphasises the value of sampling from the lower gut in addition to the respiratory tract when attempting isolation of IBV from the field (Cook, 1984). Diarrhoea was seen in broilers until 14 days p.i. which could have been associated with the activity of the virus in the lower gut, although histopathology was not done in this tissue.

Generally, it has been assumed that the caecal tonsils could be an important site for persistence of IBV, since virus has been recovered from this tissue for prolonged periods (Cook, 1968; Alexander and Gough, 1977; Alexander et al., 1978). Lucio and Fabricant (1990) also isolated virus most frequently from caecal tonsils. This variant virus was isolated from caecal tonsils only intermittently. However, *in-vitro* studies using tissue explants from a variety of gut regions have shown that not all IBV strains multiply in caecal tonsils (Bhattacharjee, 1994).

With regard to the kidneys, whilst virus could not be recovered on days 10 and 14 p.i. from SPF chickens, a high titre was recorded on day 21 p.i. On the occasions when no

virus was detected, IgM antibodies were found in the glomeruli of the kidneys. Since the birds were kept in strict isolation and virus could not be isolated on these days, it is reasonable to presume that antibodies prevented virus isolation on these occasions. Chong and Apostolov (1982) also reported Ig deposits in the glomerular basement membrane after IBV infection. In the broilers such complexes were seen from day 7 p.i. onwards and no virus was recovered from that time, but no reappearance of the virus occurred till day 28 p.i.

In one of the first descriptions of an outbreak from which this variant IBV was isolated, Gough et al. (1992) reported gross pectoral muscle changes. Similar but milder changes were reproduced experimentally with our isolate, but only in six-week old broilers. In the experimental infection, it appears that the fibres of the affected muscle were not damaged, since there was no significant increase in serum CK levels and no histological changes were observed. Though the pathogenesis of this muscle change could not be elucidated in the present study, it is tempting to speculate that it could occur as a result of immune complex deposition in the capillary walls of the muscle. This most unusual manifestation of IBV infection deserves further study, including an examination of the levels of other enzymes.

It has been shown that local replication of Newcastle disease virus (NDV) in the Harderian gland stimulates lachrimal NDV-specific IgA (Russell, 1993). This seems to be true for IBV also since virus was recovered from the Harderian gland until day 7 p.i. Davelaar et al. (1982) were able to detect IBV-specific IgG and IgA antibodies in tears in response to IBV (see also Chapter 6).

In original reports (Gough et al., 1992; D.B. Pearson, P.W. Cargill, personal communications) it was reported that this variant virus caused mortalities, its spread was slow, with pens adjoining the infected ones not showing symptoms till two weeks later. Seroconversion was also slow. Under experimental conditions, however, there was no mortality in either SPF chicks or broilers. Four of five sentinels were shedding virus by day 2 post-contact with inoculated birds and positive HI titres were seen by day 5 p.i. These results indicate that spread and seroconversion following infection with this virus is similar to other IBV strains.

Cell mediated immune responses may play a critical role in the response of the chicken to IBV, since humoral immunity does not correlate with protection (Hitchner et al., 1964 ; Raggi and Lee, 1965; Winterfield and Fadly, 1972). Immunochemistry studies revealed that CD8 cells were recruited into the trachea and lungs earlier than CD4 cells and at the time of virus clearance CD8 cells were more predominant than CD4 cells. In

contrast, Janse et al. (1994) in a similar study with a nephropathogenic IBV, observed that on day 5 p.i., when they first sampled, CD4 cells outnumbered CD8 cells. Whether this discrepancy relates to the different strain of IBV is unknown. Either way, it appears that T cells play an important role in IBV infections. The B-cells were recruited later than either class of T-cells. This latter observation was in accordance with those of Janse et al. (1994) who, contended that local immunity in IB is mediated by T-cells.

Since there is evidence of replication of this virus in lymphoid tissues including the Harderian gland and bursa of Fabricius, it is reasonable to suppose that infection could result in immunosupression. Mitogens, Con A and PHA stimulate various lymphoid cell populations (Hovi et al, 1978). Since the same number of cells from different chickens in different treatment groups were added to each test well, significant variation in the mitogenic response may reflect critical changes in the ratio of various cell types or dysfunction of a specific cell type (Cloud et al., 1992). The Con A responses of the infected birds were significantly increased on days 5, 15 and 20 p.i. This may have been due to an increase in the number of T-cells during that time. There was also increased recruitment of CD8 and CD4 cells in the affected organs during that time. However, the lack of significant differences between infected and control broilers on day 10 p.i. cannot be explained. No significant depression of mitogen responses was observed on any of the days.

Recent amino acid sequence studies of the S1 spike gene have shown that members of the 793/B subgroup of variant viruses differ from some other European and North American IBV isolates by between 21-48% (Adzhar et al., 1995). In this immunopathogenesis study in SPF chicks and broilers, while the variant strain generally behaved like other IBV strains in spread and seroconversion, unusual features of infection were the muscle changes, the rapid clearance of virus from the respiratory tract of six-week old broiler chickens and a more extensive although temporally limited replication of the virus in the gut.

# CHAPTER 9

## EFFECT OF T-CELL SUPPRESSION BY CYCLOSPORIN ON PRIMARY AND PERSISTENT INFECTIONS OF INFECTIOUS BRONCHITIS VIRUS IN CHICKENS

**CONTENTS** **PAGE NUMBER**

# CHAPTER 9

# EFFECT OF T-CELL SUPPRESSION BY CYCLOSPORIN ON PRIMARY AND PERSISTENT INFECTIONS OF INFECTIOUS BRONCHITIS VIRUS IN CHICKENS

## 9.1. INTRODUCTION

The mechanisms of protective immunity in chickens infected with avian IBV are still not fully understood (Darbyshire and Peters, 1984). Since the trachea is a primary site of virus replication, it has been suggested that local immunity is of fundamental importance in protection against IBV (Gomez and Raggi, 1974). However, systemically induced immune responses to IBV have been shown to prevent spread of virus from the trachea to other tissues (MacDonald et al., 1981; Box et al., 1988) in addition to supporting protection at a local level (Darbyshire and Peters, 1984). Vaccination studies with IBV have always focussed on the humoral immune responses in relation to protection. Studies involving the role of cell mediated immunity in IBV infections are limited (Cook et al., 1991a; Cook et al., 1992). A positive correlation between IBV-specific lymphoproliferative responses and resistance to challenge has been shown (Timms and Bracewell, 1981) and recruitment of T-cells in IBV-infected trachea, lung and kidney has been demonstrated by immunostaining (Janse et al., 1994; see Chapter 8). Wakenell et al. (1995) examined the potential role of cytotoxic T-lymphocytes but could not demonstrate their presence in IBV-infected chickens.

Infection of chickens with a pool of IBV strains either alone or in combination with *Escherichia coli* produced a readily quantifiable experimental disease in which high mortality rates consistently occurred and closely resembling natural outbreaks (Smith et al., 1985). Based on mortality rates in this experimental model, several inbred lines of chickens were classified as being resistant or sensitive to IBV infections (Bumstead et al., 1989). Otsuki et al. (1990) found that although a resistant line (C) and a sensitive line (15I) were equally susceptible to infection initially, recovery was more rapid in the resistant line. Ultrastructural and histochemical studies showed that though the type of damage to the tracheal epithelium following IBV infection was similar in both the lines of chickens, lesions were more severe and longer lasting in the sensitive line (Nakamura et al., 1991). The severity and duration of clinical infection in bursectomised line C chickens were similar to those seen in the sensitive line (Cook et al., 1991a). However no increase in mortality

was observed, in contrast to high levels of mortality recorded in line 15I chickens. Comparisons of secretory antibody responses between the two lines of chickens revealed increased local antibody secretion in the saliva and lachrymal fluid of the resistant line (Cook et al., 1992). However, the role of cell mediated immunity in the resistance of chickens to IBV infections has not been investigated.

Although IB is generally considered as an acute respiratory disease, prolonged virus excretion has been reported (Cook, 1968; Alexander et al., 1978; Chong and Apostolov, 1982). When day-old chicks were infected with an enterotropic IBV, strain G, faecal excretion could not be detected beyond day 35 p.i. but when the birds reached sexual maturity, re-excretion occurred (Jones and Ambali, 1987). Virus re-excretion could not be induced earlier by hormone injections (Ambali and Jones, 1991b) but occurred after T-cell suppression by cyclosporin (CSP) (Bhattacharjee et al., 1995).

CSP, a selective T-cell immunosuppressant drug, depresses cell-mediated immunity in chickens, causing prolonged skin graft survival, depressed proliferative responses in mitogen stimulated lymphocytes and decreased wattle responses to injected antigen (Hill et al., 1989). CSP prevents the synthesis of cytokines by T-cells by blocking a late stage in the signalling pathway initiated by the T-cell receptor. This especially affects the production of interleukin-2 (IL-2), hence T-cell proliferation is affected. As a consequence, all cell-mediated immune responses driven by cytokines, especially IL-2 dependent functions which include T-helper activities, cytotoxicity, natural killer cell activity and antibody-dependent cell cytotoxicity are depleted after CSP treatment (Schreiber and Crabtree, 1992). This drug has been extensively used to study the importance of cellular immunity in a variety of infections affecting avian species such as reovirus (Hill et al., 1989), turkey haemmorhagic enteritis virus (Suresh and Sharma, 1995) and marble spleen disease virus of pheasants (Fitzgerald et al., 1995).

The aim of the present study was to elucidate the role of T-cells in primary and persistent IBV infections by comparing the pathogenesis of IBV infections in normal and CSP-induced T-cell suppressed chickens.

## 9.2. Materials and Methods

### 9.2.1. Chickens

SPF eggs of white leghorn (WLH) chickens were obtained from a commercial source and those of brown leghorn (BLH) chickens were obtained from the Institute of Animal Health, Compton. They were hatched in our laboratory and the chicks maintained in complete isolation. They were fed and watered *ad-libitum.*

### 9.2.2. Viruses

Chicks were inoculated intra-nasally (i.n.) with IBV using either the Massachusetts (M41) strain (Ambali and Jones, 1991a) or a pool of ten virulent IBV strains all belonging to the Massachusetts serotype (IBV pool) (Smith et al., 1985).

### 9.2.3. Monoclonal antibody

Mabs to the nucleocapsid and matrix proteins of IBV strain M41 (Naqi, 1990) were obtained from Dr S. A. Naqi of the New York State College of Veterinary Medicine, Ithaca, New York, USA and used for the detection of IBV antigen by IP staining on cryostat sections of tracheas.

### 9.2.4. Hyperimmune serum

Hyperimmune serum against IBV strain G (El Houadfi et al.,1986) was used for the detection of group-specific antigen on unfixed tracheal rings (Chapter 3).

### 9.2.5. Tracheal organ culture

TOC prepared from 19-day old embryonated SPF chicken eggs following the method detailed in Chapter 3 were used for virus isolations and titrations.

### 9.2.6. T-cell suppression

T-cell suppression was induced by injections of CSP, 100mg per ml maize oil ("Sandimmun", Sandoz Pharmaceuticals, Surrey, England). The drug was given intra-muscularly at a dose of 100 mg per kg body-weight every three days. T-cell suppression was assessed by the measurement of lymphoproliferative responses to Con A and PWM using whole blood mitogen assays.

### 9.2.7. Mitogen assays

Proliferation of whole blood cells in response to stimulation with Con A and PWM was assessed following the method described in Chapter 7 except that the cultures were incubated for 96 hours. The optimum concentrations of mitogens were pre-determined and the same batch of the mitogen was used for each experiment. Low levels of $^{3}H$ thymidine uptake always occured in unstimulated cultures. Specific responses to mitogens were therefore, calculated as the difference (D) between counts per minute (cpm) of mitogen-stimulated and unstimulated cultures (Schultz, 1982). All values were first converted into their square roots before calculating D values (Woldehiwet, 1987).

### 9.2.8. Virus isolation and titrations

Aseptically collected tissues from euthanised birds were processed following the method of Cook et al. (1991a) and used for virus isolations and titrations (Chapter 3). A minimum of three passages were given in TOC and the presence of virus assessed by ciliostasis. Ciliostatic virus was identified by IF staining of unfixed TOC using hyperimmune serum against IBV strain G (Chapter 3). All the tissue samples positive for virus were titrated in TOC either individually or as pooled samples following the procedure described in Chapter 3.

### 9.2.9. Histopathology

Samples of tracheas collected in 10% neutral buffered formalin were processed by conventional methods and the sections stained with haematoxylin and eosin. The histopathological changes in the tracheal sections were scored according to the procedure described by Nakamura et al. (1991).

### 9.2.10. Immunostaining

Pieces of tracheas were collected in OCT compound (Miles Inc., Elkhart, Indiana, USA), frozen in liquid nitrogen and stored at $-70^{0}C$. 5μm thick sections were cut using a cryostat, picked onto poly-L-lysine coated slides (Sigma) and fixed in acetone for 10 minutes. IBV antigen was detected by IP staining using Mab against IBV strain M41 following the procedure described by Janse et al. (1994).

### 9.2.11. Serology

Sera were examined for VN antibodies to IBV strain M41 (100 $CD_{50}$) in TOC following the method described in Chapter 3.

### 9.2.12. Experimental Designs

A series of four experiments was performed.

#### 9.2.12.1. Effect of CSP on primary IBV infections

##### 9.2.12.1a. Experiment 1: Infection of two-week-old WLH chickens with IBV strain M41

Groups of two-week-old WLH chickens were infected i.n. with 100 µl of TOC medium containing 4.5 $\log_{10}$ $CD_{50}$ of IBV strain M41. CSP was injected on day 11 (three days prior to virus infection), day 0 (on the day of virus infection) and every third day thereafter till day 15 p.i. Heparinised whole blood collected before CSP injections, from three birds in each group was used in mitogen assays to assess lymphoproliferative responses to Con A and PWM. Chicks were examined regularly for clinical signs of infection. Samples of trachea, lung, kidney, ileum, caecal tonsils and rectum were collected from three birds in each group on days 3, 6, 9, 12 and 15 p.i. and virus recovery attempted in TOC. All the samples positive for virus isolations were titrated individually in TOC. Sections of tracheas collected in 10% formalin were stained by haematoxylin and eosin and the lesions scored. Cryostat sections of frozen tracheas were used for detection of IBV antigen by immunostaining. Serum collected from three birds on day 18 p.i. were examined for VN antibodies.

##### 9.2.12.1b. Experiment 2: Infection of two-week-old BLH chickens with the IBV pool

Groups of two-week-old BLH chickens were infected i.n. with the IBV pool. The virus titres of the ten different viruses in the pool varied from 2.75 to 4.75 $\log_{10}$ $CD_{50}$ in 0.1 ml. The chicks were treated with CSP as detailed above. Chickens were examined regularly for clinical symptoms. Heparinised blood collected from three birds in each group was used for measuring lymphoproliferative responses to Con A. Samples of trachea, lung and kidneys were collected from three birds in each group on days 3, 6, 9, 12 and 15 p.i. for virus isolations individually. All the samples positive for virus isolations were pooled for virus titrations. Serum collected from three birds on day 15 p.i. were used in VN tests against IBV strain M41.

### 9.2.12.2. Effect of CSP on persistent IBV infections

#### 9.2.12.2a. Experiment. 3: Virus re-excretion from WLH chickens infected with IBV strain M41 when two-weeks old

Chickens infected with IBV M41 at two-weeks old (Experiment 1) were treated with CSP beginning five weeks p.i. and every three days for 15 days. Heparinised blood was collected to assess T-cell suppression. Samples of trachea, lung, kidneys and caecal tonsils were collected from three birds on days 3, 6, 9, 12 and 15 post-CSP treatment. Similar tissues were also collected from age-matched IBV-infected but untreated chickens and from uninfected birds. Virus recovery was attempted in TOC.

#### 9.2.12.2b. Experiment. 4: Virus re-excretion from WLH chickens infected with IBV strain M41 when day-old

Following infection of day-old WLH chickens with 4.5 $\log_{10}$ $CD_{50}$ of IBV strain M41, samples of trachea, lung, kidneys and caecal tonsils were collected on days 3, 6, 9, 12, 15, 21, 28 and 35 p.i. for virus isolations in TOC. After the birds had recovered from the acute phase of the disease, CSP injections were given from 5 weeks p.i. every three days in an attempt to induce re-excretion of virus. Heparinised blood was taken to monitor T-cell deficiency. Samples of trachea, lung, kidney and caecal tonsils were collected on days 3, 6, 9, 12 and 15 days post-CSP treatment for virus isolations.

### 9.2.13. Statistical Analysis

The D values between experimental groups and within specific groups over time were compared using analysis of variance procedure and Bonferroni t-tests. The virus titres and serum neutralisation titres of different groups of birds were compared by students 't' test. The mortality and the total number of virus isolations between different groups of chickens were compared by the chi-square test. The tracheal lesion scores were compared by the non-parametric Mann-Whitney U-test. A p value of less than 0.05 was considered statistically significant.

## 9.3. RESULTS

### 9.3.1. Effect of CSP on primary IBV infections

#### 9.3.1.1. T-cell suppression

In these experiments CSP was used to induce selective T-cell deficiency. The administration of CSP every third day was not associated with any overt toxicity in the inoculated chickens. All the treated birds were clinically normal and no mortality was seen in control WLH birds given CSP. The D values differed significantly between experimental groups as well as over time in some groups. In Experiment 1 with WLH chickens, comparison of mean D values to Con A, by day between the four experimental groups showed that both the control + CSP and IBV + CSP groups had significantly lower values compared to the control group on days 3, 6 and 15 days p.i. and compared to the IBV group on all days except day 3 p.i. (Table 9.1). Compared to the control group, the IBV group had a significantly lower D values on day 3 p.i. and higher values on days 9, 12 and 15 p.i. No differences were seen between the control and IBV groups treated with CSP except on day 12 p.i.

**Table 9.1. D values (mean ± SEM) to Con A of WLH chickens infected with IBV strain M41 and/or treated with CSP.**

| Group | Days post-infection | | | | | | |
|---|---|---|---|---|---|---|---|
| | -3* | 0 | 3 | 6 | 9 | 12 | 15 |
| Control | 40.3 ± 14.3 | 64.4 ± 10.7 | 77.1 ± 21.5$^{a}$ | 60.1 ± 11.1$^{a}$ | 27.1 ± 5.1$^{a}$ | 42.9 ± 12.3$^{a}$ | 26.7 ± 2.8$^{a}$ |
| Control + CSP | | 38.1 ± 17.6 | 2.7 ± 0.5$^{b}$ | 1.2 ± 1.0$^{b}$ | 1.7 ± 2.8$^{a}$ | 4.1 ± 1.2$^{a}$ | 3.5 ± 1.2$^{b}$ |
| IBV | | | 30.2 ± 7.1$^{b}$ | 47.3 ± 4.9$^{a}$ | 79.9 ± 15.3$^{b}$ | 60.7 ± 10.0$^{b}$ | 59.0 ± 8.1$^{c}$ |
| IBV + CSP | | | 3.7 ± 1.7$^{b}$ | 1.8 ± 1.4$^{b}$ | 2.6 ± 1.0$^{a}$ | 3.2 ± 2.1$^{c}$ | 2.7 ± 1.9$^{b}$ |

Values with different superscripts between groups differ significantly.
* : chickens were treated with CSP from day -3 i.e.three days prior to virus infection (on day 0) and every three days thereafter.

Comparisons of D values over time showed that both control and IBV groups treated with CSP had significantly reduced mean values starting from day 3 p.i., while the control and IBV groups showed no differences in D value over time. In Experiment 2 with BLH chickens, the mean D values to Con A of the IBV-infected group were significantly lower

compared to controls only on day 6 p.i. The IBV + CSP group had significantly reduced D values on all days compared to controls and IBV-infected group (Table 9.2).

**Table 9.2. D values (mean ± SEM) to Con A of BLH chickens infected with the IBV pool and/or treated with CSP.**

| Group | Days post-infection | | | | |
|---|---|---|---|---|---|
| | 3 | 6 | 9 | 12 | 15 |
| Control | 20.0 ± 7.1[a] | 68.4 ± 7.5[a] | 101.9 ± 12.5[a] | 46.6 ± 14.0[a] | 74.7 ± 5.5[a] |
| IBV | 1.3 ± 1.1[a] | 28.1 ± 4.3[b] | 54.2 ± 2.4[a] | 42.5 ± 10.7[a] | 62.8 ± 0.7[a] |
| IBV + CSP | 0.1 ± 0.5[b] | 0.3 ± 0.8[c] | 5.7 ± 5.3[b] | -0.3 ± 1.6[b] | -0.5 ± 0.5[b] |

Values with different superscripts between groups differ significantly.

Within groups, the D values of control birds on day 9 p.i. was significantly different from those on days 3 and 12 p.i. and the day 15 p.i. value was higher than the day 3 one. The D value on day 3 p.i. in the IBV-infected group was significantly lower than those on days 9, 12 and 15 p.i. and the D value on day 15 was higher than that on day 6. No differences were seen in the values of the IBV + CSP group over time.

The D values to PWM revealed no differences between any groups on any occasion (Table 9.3). Within the group, the mean D value of the IBV group on day 9 p.i. was higher than those on days 3, 12 and 15 p.i. while in the IBV + CSP group the value on day 9 p.i. was significantly higher compared to those on days 12 and 15 p.i.

**Table 9.3. D values (mean ± SEM) to PWM of WLH chickens infected with IBV strain M41 and/or treated with CSP.**

| Group | Days post-infection | | | | | | |
|---|---|---|---|---|---|---|---|
| | -3 | 0 | 3 | 6 | 9 | 12 | 15 |
| Control | 20.4 ± 3.1 | 19.3 ± 4.0 | 21.1 ± 4.3 | 19.2 ± 4.4 | 17.0 ± 1.6 | 8.9 ± 2.2 | 15.7 ± 4.7 |
| Control + CSP | | 27.4 ± 8.0 | 20.1 ± 5.7 | 19.2 ± 3.1 | 16.0 ± 3.9 | 11.9 ± 1.2 | 11.7 ± 1.5 |
| IBV | | | 23.9 ± 5.3 | 29.0 ± 5.2 | 49.1 ± 5.2 | 11.0 ± 3.7 | 12.0 ± 1.7 |
| IBV + CSP | | | 23.3 ± 1.6 | 13.8 ± 1.7 | 47.6±21.1 | 6.3 ± 3.1 | 5.7 ± 1.8 |

### 9.3.1.2. Clinical signs and mortality

The results summarised in Table 9.4 show that following inoculation of BLH chickens with the IBV pool, clinical signs were more severe and longer lasting in the T-cell suppressed chickens than in the intact birds. Respiratory signs were less severe following inoculation of WLH chicks with IBV strain M41 in both groups of birds, but, the T-cell deficient birds showed slightly more severe clinical disease. No clinical signs were observed in the control chickens.

**Table 9.4. Clinical signs of respiratory infection in normal and CSP-treated WLH or BLH chicks following inoculation with IBV.**

| Days post-infection | Clinical signs following inoculation with | | | |
|---|---|---|---|---|
| | M41 in WLH | | IBV Pool in BLH | |
| | intact | CSP-treated | intact | CSP-treated |
| 2 | ++ | ++ | ++ | ++ |
| 5 | ++ | +++ | ++ | +++ |
| 7 | ± | ++ | + | +++ |
| 9 | - | + | - | +++ |
| 12 | - | - | - | ++ |
| 15 | - | - | - | - |

+++, ++, +, ± severity of respiratory signs
- no respiratory signs

The mortalities following i.n. inoculations of IBV are shown in Table 9.5. Those in CSP treated WLH chickens inoculated with IBV strain M41 was 18.4% against 2.2% in the intact group. None of the BLH chickens infected with the IBV pool died while after CSP treatment 43% of BLH chicks succumbed to infection. No deaths were recorded in uninfected control WLH and BLH chickens nor in control WLH birds treated with CSP.

**Table 9.5. Mortalities during 15 days p.i. in WLH and BLH chickens infected with IBV strain M41 or IBV pool and/or treated with CSP.**

| Group | IBV strain M41 in WLH | | IBV pool in BLH | |
|---|---|---|---|---|
| | No. died/ No. infected | Percent mortality | No. died/ No. infected | Percent mortality |
| IBV | 1/45 | 2.2 | 0/18 | 0 |
| IBV + CSP | 7/38 | 18.4[a] | 13/30 | 43[a] |
| Control | 0/22 | 0 | 0/12 | 0 |
| Control + CSP | 0/16 | 0 | ND | ND |

a : indicates significant differences compared to IBV group by chi-square test.
ND : not done

### 9.3.1.3. IBV recovery from tissues

Table 9.6 shows the results of virus isolations and titrations from intact and CSP-treated WLH chickens. No significant differences were found in the total numbers of virus isolations between intact and T-cell suppressed WLH chicks. The mean titre for the tracheas of CSP-treated birds was significantly higher than that for intact chicks on day 6 p.i., although the rate of virus clearance was the same. For the other tissues, differences in titres between treated and untreated groups were small.

Table 9.7 shows the results for BLH chickens. The number of virus isolations from the lungs of CSP-treated birds was significantly higher than untreated birds. In general, the number of virus isolations was higher in the IBV pool-infected chickens than in the M41-infected WLH chickens.

Virus titres in the tracheas of both groups of birds were similar, while the virus persisted for at least 6 days longer in the lungs of immunosuppressed chicks. Virus titres in the kidneys of T-cell suppressed chickens were always 1 to 3 $\log_{10}$ $CD_{50}$ higher than in intact birds, although both groups were negative for virus by day 15 p.i.

**Table 9.6. Virus isolations and mean titres (shown in brackets) from normal and CSP-treated WLH chickens infected with IBV strain M41.**

| Tissue | Group | Days post-infection 3 | 6 | 9 - 15 | Total[b] |
|---|---|---|---|---|---|
| Trachea | IBV | 2[a] (4.0 ± 0.354) | 2 (2.5 ± 0) | 0 | 4 |
| | IBVC | 1 (3.75) | 3 (3.58 ± 0.14)[c] | 0 | 4 |
| Lung | IBV | 0 | 0 | 0 | 0 |
| | IBVC | 1 (2.5) | 1 (2.5) | 0 | 2 |
| Kidney | IBV | 0 | 2 (3.13 ± 0.88) | 0 | 2 |
| | IBVC | 0 | 1 (4.25) | 0 | 1 |
| Ileum | IBV | 0 | 3 (2.5 ± 0) | 0 | 3 |
| | IBVC | 0 | 2 (2.88 ± 0.53) | 0 | 2 |
| Caecal tonsil | IBV | 0 | 1 (2.5) | 0 | 1 |
| | IBVC | 0 | 1 (2.5) | 0 | 1 |
| Rectum | IBV | 0 | 2 (2.5 ± 0) | 0 | 2 |
| | IBVC | 0 | 2 (2.88 ± 0.53) | 0 | 2 |

IBV : Intact two-week old chickens infected with IBV
IBVC : CSP-treated two-week old chickens infected with IBV
a : no. of samples positive for virus isolations in TOC out of three samples tested.
b : total no.of samples positive for virus isolations out of 15 samples tested.
c : indicates significantly different virus titre ($log_{10}$ $CD_{50}$) compared to normal chicken infected with IBV.

**Table 9.7. Virus isolations and titres (shown in brackets) from normal and CSP-treated BLH chickens infected with the IBV pool.**

| Tissue | Group | Days post-infection 3 | 6 | 9 | 12 | 15 | Total |
|---|---|---|---|---|---|---|---|
| Trachea | IBV | 3 (3.75) | 3 (4.25) | 2 (2.5) | 0 | 0 | 8 |
| | IBVC | 3 (4.5) | 3 (3.75) | 2 (2.5) | 0 | 0 | 8 |
| Lung | IBV | 2 (3.25) | 0 | 0 | 0 | 0 | 2 |
| | IBVC | 3 (3.5) | 2 (2.5) | 2 (3.5) | 0 | 0 | 7[a] |
| Kidney | IBV | 1 (2.0) | 2 (2.5) | 2 (2.5) | 3 (3.0) | 0 | 8 |
| | IBVC | 3 (4.25) | 3 (5.5) | 3 (4.5) | 2 (4.0) | 0 | 11 |

a : indicates significant differences in the number of virus isolations from similar tissues of intact chickens infected with IBV (chi-square test , $p<0.05$). Legend: see table 9.6

### 9.3.1.4. Histopathology and Immunostaining

Table 9.8 shows the histopathological changes and IBV antigen detection in the tracheal sections from WLH chickens (Experiment 1). Although the lesion scores were higher in the CSP-treated chickens, they were not statistically significant (Mann Whitney U test, $p >0.05$). No differences were observed in the numbers of birds positive for IBV antigen in either group.

### 9.3.1.5. Serology

The mean ± s.d. $\log_2$ VN titre of sera derived from CSP-treated BLH birds at 15 days after being infected with the IBV pool was 9.417 ± 0.804, which was significantly higher than the mean titre of intact chickens (5.83 ± 0.577). The mean 18 day p.i. titres of CSP-treated WLH birds infected with IBV strain M41 was 8.08 ± 1.377, compared to the titre of 6.58 ± 0.629 for intact WLH chickens. This increase was not statistically significant (t- test, $p> 0.05$).

**Table 9.8. Histology and immunochemistry in tracheas of normal and CSP-treated WLH birds inoculated with IBV strain M41.**

| | IBV | | | | | | | IBV + CSP | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DPI | CI | De | Hy | Mu | He | Ly | IP | CI | De | Hy | Mu | He | Ly | IP |
| 3 | 3 [a] | 1.3 | 1.0 | 1.3 | 1.0 | 0 | 2 | 3.0 | 3.0 | 0 | 3.0 | 1.0 | 0 | 1 |
| 6 | 2.7 | 1.7 | 2.3 | 2.3 | 0.3 | 2.0 | 2 | 3.0 | 3.0 | 2.0 | 3.0 | 1.0 | 2.0 | 3 |
| 9 | 2.3 | 0.3 | 0.7 | 1.0 | 0 | 0.7 | 0 | 2.3 | 1.0 | 2.0 | 1.7 | 0 | 2.0 | 0 |
| 12 | 0 | 0 | 0.3 | 0 | 0 | 1.0 | 0 | 0.3 | 0.7 | 1.0 | 0.7 | 0 | 0.7 | 0 |
| 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.7 | 0 | 0 | 0.7 | 0 |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.0 | 0 |

DPI : days post-infection
CI : loss of cilia by tracheal epithelial cells
De : Degeneration of tracheal epithelial cells
Hy : Hyperplasia of tracheal epithelial cells
Mu : Depletion of mucus-secreting cells
He : Heterophilic infiltration
Ly : Lymphocytic infiltration
IP : No. positive for virus antigen by IP test
a : Mean severity index (0, 1, 2 and 3 = no, mild, moderate and severe lesions, respectively in 3 birds each time.

### 9.3.2. Effect of CSP on persistent IBV infections

Two-week old chickens were infected with 4.5 $\log_{10}$ $CD_{50}$ of IBV strain M41 as in Experiment 1 and from five weeks p.i., CSP injections were given every third day for 15 days in an attempt to induce viral re-excretion. No virus was recovered from any of the tissue samples on any occasion although the mitogen responses of these birds to Con A were significantly reduced compared to uninfected controls (Table 9.9).

**Table 9.9.D values (mean ± SEM) to Con A of WLH chickens infected when 2 weeks old with IBV strain M41 and treated with CSP from 49 days of age.**

| Group | Days post-infection | | | | |
|---|---|---|---|---|---|
| | 52 | 55 | 58 | 61 | 64 |
| Control | 29.9 ± 3.1 | 37.5 ± 8.6[a] | 28.0 ± 1.5 [a] | 19.2 ± 0.4[a] | 32.0 ± 8.9[a] |
| IBV + CSP | 13.2 ± 5.5 | 11.0 ± 2.2[b] | -0.38 ± 0.4[b] | 2.8 ± 1.2[b] | 1.1 ± 0.3[b] |

Values with different superscripts between groups differ significantly in t-test, $p<0.05$.

In order to determine if the age at infection is important for the virus to persist, day-old chicks were infected with the same dose of IBV strain M41 and CSP injections were given from 5 weeks p.i. During the acute phase of infection, mortality in these day-old infected chicks was 3.6% (2 / 55) and Table 9.9 shows the results of virus isolations. The total number of virus isolations till day 15 p.i. from these birds (29 positive out of 60 samples) was higher than that from birds infected when two-weeks old with the same virus (7 of 60 samples). No virus was recovered from the tissues sampled between days 15 and 35 p.i. from which time CSP injections were given every third day. This treatment regimen caused a significant reduction of lymphoproliferative responses to Con A compared to infected but untreated chickens or uninfected control chickens on all occasions (Table 9.10). After three injections of CSP, virus was first re-isolated on day 44 p.i. from the kidneys of one bird (Table 9.9).

**Table 9.9. Virus isolations in TOC from WLH chickens infected with IBV strain M41 when day-old and treated with CSP from 35 days of age.**

| Tissues | Days post-infection | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | 6 | 9 | 12 | 15 - 35 | 38 | 41 | 44 | 47 | 50 |
| Trachea | 3[a] | 3 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Lung | 2 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 1 | 0 |
| Kidney | 2 | 1 | 2 | 3 | 0 | 0 | 0 | 1 | 3 | 1 |
| Caecal tonsil | 1 | 1 | 1 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |

a : no. of samples positive for virus out of three samples tested.
b : CSP injections were given from day 35 p.i., every three days in an attempt to induce re-excretion of virus.

**Table 9.10. D values (mean ± SEM) to Con A of WLH chickens infected with IBV strain M41 when day-old and treated with CSP from 35 days of age.**

| Group | Days post-infection | | | | |
|---|---|---|---|---|---|
| | 38 | 41 | 44 | 47 | 50 |
| Control | 47.9± 4.1[ab] | 72.0 ± 15.8[a] | 48.1 ± 0.4[a] | 39.4 ±10.4 [a] | 36.8 ± 4.0[a] |
| IBV | 95.3 ± 25.6[a] | 65.4 ± 5.0[a] | 44.7 ± 8.4[a] | 35.4 ± 5.7[ab] | 71.9 ± 14.4[b] |
| IBV + CSP | 14.1 ± 1.0[b] | 5.1 ± 2.6[b] | 7.6 ± 2.1[b] | 2.0 ± 1.0[b] | 6.6 ± 2.6[a] |

Values with different superscripts between groups differ significantly.

In all, virus was recovered from 7 / 60 samples tested. Although one sample each of trachea and lung was positive, these tissues were not positive when the kidneys of the same birds were negative. Virus could not be isolated from the caecal tonsils of any of these birds. None of the birds which re-excreted virus showed any apparent clinical symptoms. No virus was recovered from similar tissue samples obtained from age-matched infected but untreated chickens or from uninfected controls.

## 9.4. DISCUSSION

The injection of chickens every three days with CSP caused a significant reduction in response to the T-cell mitogen Con A. The proliferative response to a B and T cell mitogen, PWM, was not altered significantly. PWM has been shown to stimulate mainly B-cells and only a subset of T-cells (Vainio and Ratcliffe, 1984). Although it would have been ideal to use a B-cell mitogen like lipopolysaccharide to determine the selectivity of T-cell suppression, in our hands, the responses to this mitogen in whole blood cultures were very low, a finding also reported with chicken lymphocytes by Vainio and Ratcliffe (1984). Nowak et al. (1982) have shown that CSP acts as a selective T-cell suppressor in chickens. Furthermore, Suresh and Sharma (1995) have shown that similar injections of CSP did not decrease the humoral responses to sheep red blood cells and brucella antigens in turkeys.

The main findings in these experiments were (i) T-cells protect chickens against IBV-induced mortalities and (ii) the kidney is the site of virus persistence, but the occurrence of persistence is related to the age of infection.

In the first experiment using IBV strain M41 in WLH chicks, although virus titres in trachea, lung and kidneys were higher in the T-cell-suppressed chickens, the most marked effect was seen in the mortality rate. To confirm that T-cells prevent mortality due to IBV, a second experiment was conducted in which the pool of IBV known to cause more severe disease was used to infect a line of chickens (BLH) known to be resistant to this virus pool. The IBV pool has been shown to cause high levels of mortality in susceptible chicks either alone (Bumstead et al., 1989) or in combination with pathogenic serotypes of *E. coli* (Smith et al., 1985; Cook et al., 1986). The mortality rates in inbred lines of chickens challenged with the IBV pool varied from 3% in BLH to 47% and 83% in lines 15I and 7 respectively (Bumstead et al.,1989). Hence BLH chicks were classified as being resistant to IBV infections while lines 15I and 7 were considered sensitive.

The mortality rate in the CSP-treated BLH chickens was 43% compared to 0% in intact chickens. Thus a resistant chicken line was induced to behave like a sensitive one, in terms of mortality. Infection of bursectomised chicks of another resistant line (C) with the same IBV pool produced a more severe clinical disease and prolonged virus persistence but caused only negligible mortality (Cook et al., 1991a). Thus it appears that T-cell mediated immunity is important for the chicken in surviving the acute stages of IBV infections. This suggests that mortality in field cases of IBV depends on the virulence of the virus, genetic nature and immune status of the flock. Factors which could increase morbidity and mortality due to IBV by T-cell immunosuppression include chicken anaemia virus infection or ingestion of immunosuppressive substances like mycotoxins.

Thus although it was shown that T-cells prevent IBV-induced mortality the class of T-cells involved in these effects was not investigated here. Recently it has been suggested that cytotoxic CD8 cells are not important in IBV infections (Wakenell et al., 1995). If this is so, the data presented here would imply that CD4 cells, which act as helper cells for B-cells and secrete cytokines may play a significant role in immunity to IBV infections. Janse et al. (1994) have shown a greater increase in helper CD4 cells in the organs affected following a nephropathogenic IBV infection. Furthermore, with another member of the Coronaviridae family, mouse hepatitis virus, it has also been shown that deaths due to the virus can be prevented by adoptive transfer of CD4 splenic T-cells (Kroner et al., 1991) and delayed type hypersensitivity inducer T-cells (Stohlman et al., 1986).

In the present study, it was observed that IBV strain M41 caused depression in mitogen responses to Con A on day 3 p.i.while the IBV pool was immunosuppressive on day 6 p.i. Such transient reduction in proliferative responses to another T-cell mitogen, PHA has been reported following virulent IBV infection (Wakenell et al., 1995). The mortality in day-old chicks infected with IBV strain M41 was 4% compared to no mortality in chicks infected with the same virus when two weeks old. This may be related to the increased resistance to IBV-induced mortalities as chickens become older (Smith et al., 1985).

The most marked effect of T-cell suppression on virus titres was seen in the kidneys of BLH chicks. Interestingly, both the groups of chickens were able to clear the virus equally efficiently, unlike in chicks incapable of antibody production where prolonged virus persistence was reported (Cook et al., 1991a). Although NIBV are recognised, it appears that even with the more respiratory strains of IBV, kidney damage could be severe in immunosuppressed birds and could be a reason for the mortalities

observed. Unfortunately in this study, kidneys were not examined histologically for the severity or extent of lesions. Renal lesions suggestive of toxicity after CSP administration has been described in humans (White, 1986). In this study, no gross changes were seen in the kidneys of control birds given CSP. In another study but with reoviruses, no histological renal lesions were observed in chickens treated with CSP (Hill et al., 1989).

The serum neutralisation titres of CSP treated WLH and BLH chicks were higher than in intact birds. Arnold and Holt (1995) reported a 5% increase in the B-cell population following CSP treatment of chickens. The greater humoral immune response could also have been elicited by the higher virus titres in the lungs and kidneys of T-cell immunosuppressed birds. A similar increase in serum IgG titres has been reported in chickens treated with another T-cell suppressor drug, dexamethasone, and infected with *Eimeria mivati* (Isobe and Lillehoj, 1993).

Prolonged IBV excretion has been reported from kidneys (Chong and Apostolov, 1982) and/or caecal tonsils (Alexander et al.,1978; Cook, 1968), which have been thought to be the possible sites of virus persistence. In the present study, CSP treatment of birds infected with IBV strain M41 when two-weeks old did not induce any re-excretion of virus. In a similar study done with day-old chicks infected with an enterotropic variant, IBV strain G, CSP treatment induced re-excretion of virus as assessed by virus isolations in tracheal and cloacal swabs (Bhattacharjee et al., 1995).

In order to determine whether age at infection determines whether virus will be persistent, Experiment 4 was performed. The results confirm that infection at day-old does lead to persistence, not only with enterotropic strains of IBV (Bhattacharjee et al., 1995), but also with 'conventional' IBV strains like M41. Virus re-excretion was mainly from the kidneys and not from the caecal tonsils. From the results of this study, it may be reasonable to conclude that kidneys, rather than caecal tonsils, is the primary site of virus persistence. The target cells of the virus in the kidneys are the tubular epithelium (Chong and Apostolov, 1982) which provides an ideal site for virus persistence because of its immunologically privileged nature (Mimms, 1988). In the previous study (Bhattacharjee et al., 1995), the shedding of the virus from renal tubules could have accounted for a higher proportion of virus isolations from the cloacal swabs than from tracheal swabs. CSP-treated BLH chicks infected with the IBV pool had markedly higher virus titres in the kidneys. CSP treatment of chickens infected at day-old, could have increased the replication of the persisting virus and thus the chances of virus recovery.

Virus re-excretion by T-cell suppression of persistently infected chicks with no clinical signs may have several practical implications in the epidemiology of the disease. In particular, the excretion of virus in the faeces by birds without apparent clinical signs, would increase the probability of spread from infected to susceptible flocks in close proximity.

# CHAPTER 10

## A STUDY OF CERTAIN NON-SPECIFIC FACTORS ASSOCIATED WITH IMMUNITY TO INFECTIOUS BRONCHITIS VIRUS INFECTION IN CHICKENS

**CONTENTS** **PAGE NUMBER**

# CHAPTER 10

# A STUDY OF CERTAIN NON-SPECIFIC FACTORS ASSOCIATED WITH IMMUNITY TO INFECTIOUS BRONCHITIS VIRUS INFECTION IN CHICKENS

## 10.1. INTRODUCTION

In this Chapter, the role of certain non-specific defence mechanisms in immunity to IBV infections was investigated, with special reference to that of heterophils.

Neutrophils constitute the 'first line of defence' against infectious agents or 'non-self' substances that penetrate the body's physical barriers. Avian heterophils are functionally equivalent to mammalian neutrophils (Brune and Spitznagel, 1973). Neutrophils destroy microorganisms by both oxygen-dependent and oxygen-independent mechanisms. Avian heterophils lack the enzymes alkaline phosphatase, catalase and myeloperoxidase responsible for oxygen-dependent killing (Breton-Gorius et al., 1978). Hence the avian cells rely more on oxygen-independent mechanisms that involve lysozyme and cationic proteins like acid hydrolases and cathepsin.

Heterophils are usually viewed as being important in fighting bacterial infections and their importance has been well recognised in a variety of bacterial and fungal diseases affecting poultry (Chang and Hamilton, 1979; Rossack et al., 1981; Andreasen et al., 1990; Kogut et al., 1993). However, there are reasons for believing that heterophils may also contribute to host defence against virus attack. It is known that certain virus infections can lead to an increase in blood heterophil count and predominant heterophil infiltration has been reported to occur at sites of infection with viruses such as IBV (Purcell and McFerran, 1972). Nevertheless, the importance of these cells in viral infections affecting poultry is unknown. Recently, it has been shown that neutrophils play a significant role in controlling the replication and spread of Herpes simplex virus infections in mice (Tumpey et al., 1996).

Heterophils are the major early inflammatory cells in tracheal, kidney and oviduct lesions caused by IBV (Purcell and McFerran, 1972; Chong and Apostolov, 1982; Crinion et al., 1971a). They were found to be the most prevalent inflammatory cells in the respiratory lavage fluids collected from normal two and six-week old SPF chickens (Fulton et al., 1990) and their numbers increased dramatically in IBV infected chickens

(Fulton et al., 1993). However, the role of these cells in limiting virus replication or in the development of lesions caused by IBV is not known.

Two anti-tumour drugs, cyclophosmamide and 5-fluorouracil (5 FU) have been used to deplete neutrophils in mice *in-vivo* (McIntyre et al., 1989 ; van der Meer et al., 1988). These chemicals have also been shown to exert a similar effect in chickens (Kogut et al., 1993; Fulton et al., 1996).

Tumour necrosis factor (TNF) is a cytokine primarily secreted by activated monocytes / macrophages, natural killer cells and lymphocytes (Cuturi et al., 1987; Mannel et al., 1980). Many infectious or inflammatory stimulants such as bacterial endotoxins, viruses and parasites, are capable of triggering TNF biosynthesis (Byrnes et al., 1993; Zhang and Lillehoj, 1995). Most of the studies involving TNF have been done in mammals. A TNF-like protein (TNLF), distinct from interleukin-1, has been described in culture supernatants of chicken macrophages stimulated with lipopolysaccharide (Klasing and Peng, 1990; Miller and Qureshi, 1992) or *Eimeria tenella* sporozoites and merozoites (Zhang and Lillehoj, 1995; Zhang et al., 1995).

The main aim of the present study was to investigate the importance of heterophils in IBV infection using 5 FU to deplete these cells *in-vivo*. In addition, the levels of lysozyme, acid phosphatase and the inflammatory cytokine TNLF were also quantitated in the sera of birds following IBV infection.

## 10.2. Materials and Methods

### 10.2.1. Chickens

SPF eggs of WLH chickens obtained from a commercial source were hatched in our laboratory and the chicks maintained in complete isolation as described in Chapter 3. They were fed and watered *ad-libitum.*

### 10.2.2. Virus

Two-week old chickens were inoculated i.n. with 4.5 $\log_{10}$ $CD_{50}$ of IBV strain M41 (Ambali and Jones, 1991a).

### 10.2.3. Monoclonal antibody

Mab to the nucleocapsid and matrix proteins of IBV strain M41 (Naqi, 1990) were used for the detection of IBV antigen by IP staining of tracheal sections. The source of the Mab is described in Chapter 9.

### 10.2.4. Tracheal organ culture

TOC prepared from 19- or 20-day old embryonated SPF chicken eggs following the method described in Chapter 3 were used for virus titrations.

### 10.2.5. Induction of heteropaenia

Heteropaenia was induced in 11-day old chickens by a single intravenous injection of 5 FU (Sigma) dissolved in pyrogen-free saline at a dose of 200 mg per kg body-weight (Kogut et al., 1993).

### 10.2.6. Peripheral blood counts

Total and differential blood counts were determined following the procedure described by Campbell (1988). Blood was diluted 1 : 200 in Natt and Herrick (1952) diluent (see Appendix) and the total number of leukocytes was counted in a haemocytometer. Simultaneously, air-dried blood smears were stained with the rapid Romanowsky stain (H. D. Supplies, Aylesbury, U.K.). Differential counts were made by counting 100 cells on each smear. Cells were identified according to the descriptions of Lucas and Jamroz (1961). Only data for lymphocytes, large mononuclear cells and polymorphonuclear cells (PMN) were collected. Because of the low numbers of eosinophils and basophils in chicks all the PMN cells counted were considered to be heterophils (Lucas and Jamroz, 1961). The absolute numbers of each cell type per cubic millimetre were calculated for each bird from the total and differential counts.

### 10.2.7. Virus titrations

Aseptically collected tissues from euthanised birds were processed following the method detailed in Chapter 3 and used for virus titrations in TOC. All the samples were titrated individually and endpoints determined by complete ciliostasis. Virus titres were expressed as $CD_{50}$.

### 10.2.8. Histopathology

Samples of tracheas collected in 10% neutral buffered formalin were processed by conventional methods and the sections stained with haematoxylin and eosin. The histopathological changes were scored according to the method described by Nakamura et al. (1991).

### 10.2.9. Immunochemistry

A streptavidin-biotin method of IP staining for virus detection was performed on paraffin embedded 5 μm thick sections, using a commercially available kit (Dako Corporation, California, USA). Staining was done according to the manufacturer's instructions with the Mab against IBV as the primary antibody.

### 10.2.10. Lysozyme levels in serum

Serum lysozyme concentrations were determined using the lyso-plate technique described by Ossermann and Lawlor (1966). Briefly, a 50 mg/ml suspension of heat-killed cells of the lysozyme-sensitive bacterium, *Micrococcus lysodeikticus* (Sigma) was set in 1 % agarose in PBS (pH 6.3). 20 µl of serum was added to 3 mm holes punched in the agar base and the resulting zones of lysis were measured after 24 hrs against known quantities of hen's egg lysozyme (Sigma) as standard. The concentration of lysozyme in the serum samples was determined by comparison with the standards and expressed in µg/ml.

### 10.2.11. Acid phosphatase levels in serum

Serum acid phosphatase levels were determined by a colorimetric method using a commercial kit (Randox Laboratories Ltd., Antrim, U.K) following the manufacturer's instructions and the results expressed in units /litre.

### 10.2.12. Serum TNLF levels

TNLF activity was determined in serum samples of control and IBV infected chickens using a murine cell line (L929) sensitive to mammalian TNF and following the procedure of Asai et al. (1993). Briefly, 3 x $10^4$ L929 cells (kindly donated by Dr. S. D. Carter, University of Liverpool, UK) in 50 µl of RPMI 1640 medium containing 25 mM HEPES and glutamax (Life Technologies, Irvine, Scotland) and supplemented with 10% foetal calf serum, 2 µg/ml actinomycin D, 1 mM sodium pyruvate, benzyl penicillin (250 IU/ml) and streptomycin (250 µg/ml) were added to each well of a 96-well plate with 50 µl of 1 : 2 dilution of serum samples in triplicate. After incubation for 24 hours at 37°C in 5% $CO_2$, the viability of target cells was determined using the 3 - (5, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) method described by Mosmann (1983) and modified by Hansen et al. (1990). Twenty microliters of 5 mg/ml solution of MTT in PBS was added to each well and the plates incubated for 4 hours at 37°C. Then, 100 µl of extraction buffer (12.5 % sodium dodecyl sulphate in 50% dimethylformamide, pH 4.7) was added. Following overnight incubation at 37°C, the absorbance was read at 570 nm in an ELISA reader (Dynatech Laboratories, UK) and specific cytotoxicity was expressed by the following formula (Zhang et al., 1995) :

$$\text{percentage cytotoxicity} = (A - B)/(A - C) \times 100,$$

where A represents the absorbance of control wells containing culture medium alone, B represents the absorbance of sample wells and C represents the absorbance of wells to which 0.5% triton X-100 was added to achieve maximum cytolysis.

### 10.2.13. Experimental Design

Groups of 11-day old SPF chickens were inoculated intravenously with 5 FU. Three days after the injection (day 0) IBV was given i.n. Peripheral blood counts were performed on three birds in each group on days -3, 0, 3, 5, 7 and 10 days p.i. Chickens were examined regularly for the presence of nasal exudate. The consistency of nasal exudate was scored on a 0 to 3 scale ; 0 for no exudate, 1 for thin clear exudate, 2 for thick and turbid exudate and 3 for purulent exudate with swollen infra orbital sinuses. Samples of trachea, lung and kidney were collected from three birds in each group on days 1, 3, 5, 7 and 10 p.i. for virus titrations. Samples of tracheas were collected in 10% formalin from three birds each, on days 3, 5, 7 and 10 p.i. for histopathological scoring and immunochemistry.

Five control and five IBV infected birds were bled on days 0, 1, 3, 5, 7 and 10 p.i. Sera separated from these samples were used to assess levels of lysozyme, acid phosphatase and TNLF.

### 10.2.14. Statistical analysis

The absolute numbers of blood cells were compared by one-way analysis of variance and Bonferroni t-test. The total numbers of chickens in each group with nasal exudate were compared by the chi-square test ($p < 0.05$). The virus titres, serum lysozyme and acid phosphatase levels were compared by the student's t-test ($p < 0.05$).

## 10.3. RESULTS

Tables 10.1 and 10.2 show the absolute numbers of heterophils and lymphocytes from 5 FU- treated and untreated chickens infected with IBV. When compared to untreated controls, heterophil counts of normal chickens treated with 5 FU were significantly decreased from day 3 post-treatment (p.t.) (day 0) to day 10 p.t. (day 7 p.i.). No significant differences were observed on day 13 p.t. (day 10 p.i.). A similar pattern was seen in 5 FU-treated chicks infected with IBV. No significant differences in the heterophil counts were seen between control and IBV-infected chicks on any of the days tested.

Within groups, no significant differences in the heterophil numbers were seen in control or IBV-infected chickens. In the control chickens treated with 5 FU, the heterophil numbers on days -3 and 10 p.i. were significantly higher than those on other days while in the IBV-infected chickens treated with 5 FU, the heterophil numbers on

day -3 p.i. were significantly higher than those on days 0, 3, 5 and 7 p.i. and the day 10 p.i.cell numbers were higher than day 5 p.i. values.

**Table 10.1. Numbers of circulating heterophils (x 10 $^3$/cu.mm) in two-week old chickens given 5 FU and/or infected with IBV**

| Groups | Days post-infection | | | | | |
|---|---|---|---|---|---|---|
| | -3 | 0 | 3 | 5 | 7 | 10 |
| Control | 5.0 ± 1.0* | 4.0 ± 1.5$^a$ | 4.4 ± 1.3$^a$ | 3.6 ± 1.5$^a$ | 4.2 ± 1.0$^a$ | 5.6± 1.1 |
| Control + 5 FU | | 1.5 ± 0.02$^b$ | 1.2 ± 0.6$^b$ | 0.3 ± 0.1$^b$ | 0.7 ± 0.2$^b$ | 5.4 ± 2.3 |
| IBV | | | 3.4 ± 0.8$^{ab}$ | 4.0 ± 1.8$^a$ | 5.3 ± 0.6$^a$ | 4.9 ± 1.7 |
| IBV + 5 FU | | | 1.1 ± 0.6$^b$ | 0.2 ± 0.1$^b$ | 1.5 ± 1.0$^b$ | 4.4 ± 2.2 |

* Each value represents the mean (± standard deviation) of heterophil counts made from three different birds on each occasion. Values with different superscripts between groups differ significantly.

When compared to untreated controls, lymphocyte counts in 5 FU-treated normal chickens were significantly reduced on days 3 and 6 p.t. (days 0 and 3 p.i.). IBV-infected chickens had lower lymphocyte counts on days 3 and 5 p.i., while 5 FU-treated chickens infected with IBV had significantly lower lymphocyte counts only on day 3 p.i. No significant differences in the lymphocyte counts were observed between any of the groups on other days.

**Table 10.2. Numbers of circulating lymphocytes (x 10 $^3$/cu.mm) in two-week old chickens given 5 FU and/or infected with IBV**

| Groups | Days post-infection | | | | | |
|---|---|---|---|---|---|---|
| | -3 | 0 | 3 | 5 | 7 | 10 |
| Control | 15.4 ± 2.8* | 15.8 ± 5.1$^a$ | 12.6 ± 1.5$^a$ | 17.2 ± 5.0$^a$ | 14.7± 1.1 | 16.7 ± 3.1 |
| Control + 5 FU | | 5.7 ± 0.8$^b$ | 4.3 ± 1.4$^b$ | 10.1 ± 2.0 | 10.6 ± 3.6 | 20.5 ± 6.3 |
| IBV | | | 7.1 ± 0.1$^b$ | 9.0 ± 1.0$^b$ | 12.2 ± 1.8 | 14.9 ± 6.1 |
| IBV + 5 FU | | | 6.3 ± 1.1$^b$ | 9.3 ± 1.4 | 13.8 ± 1.0 | 11.9 ± 1.7 |

* Each value represents the mean (± standard deviation) of lymphocyte counts made from three different birds on each occasion. Values with different superscripts between groups differ significantly.

Within groups, no significant differences were seen in the lymphocyte counts of control or IBV-infected chickens. In the control chickens treated with 5 FU, the cell counts on day -3 were higher than those on day 3 p.i. and the day 10 p.i. values were higher than day 0, 3 and 5 p.i. values. In the IBV-infected chickens treated with 5 FU, the lymphocyte numbers on day -3 were higher than those on day 0, 3 and 5 p.i. and the day 7 and 10 p.i. values were higher than on days 0 and day 3 values.

The monocyte counts did not significantly differ between treated and un-treated chickens (Table 10.3).

**Table 10.3. Numbers of circulating monocytes in two week-old chickens given 5 FU and/or infected with IBV**

| Groups | Days post-infection | | | | | |
|---|---|---|---|---|---|---|
| | -3 | 0 | 3 | 5 | 7 | 10 |
| Control | 1820 ± 189 | 707 ± 361 | 1011 ± 637 | 1560 ± 256 | 793 ± 366 | 1109 ± 511 |
| Control + 5 FU | | 586 ± 75 | 744 ± 407 | 1367 ± 277 | 681 ± 246 | 957 ± 214 |
| IBV | | | 1071 ± 132 | 801 ± 403 | 969 ± 79 | 1131 ± 557 |
| IBV + 5 FU | | | 584 ± 330 | 1239 ± 418 | 632 ± 257 | 1605 ± 990 |

It was found that the number of birds with nasal exudate was significantly higher in the 5 FU-treated and IBV-infected group (Table 10.4). However, in contrast to the thick and turbid nasal exudate seen in most of the IBV-infected normal chickens, in IBV-infected heteropaenic chickens it was consistently very thin, watery and clear. Hence, the severity scores for the exudate were much less in the heteropaenic chickens (Figure 10.1).

**Table 10.4. Presence of nasal exudate in chickens infected with IBV and/or treated with 5 FU**

| Groups | Days post-infection | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 | 9 | 10 | Total |
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | $0^a$ |
| Control + 5 FU | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | $0^a$ |
| IBV | 0 | 3 | 3 | 2 | 2 | 0 | 0 | 0 | $10^b$ |
| IBV + 5 FU | 2* | 5 | 5 | 5 | 5 | 3 | 0 | 0 | $25^c$ |

* indicates number of chickens with nasal exudate of five birds checked each time.
Values with different superscripts differ significantly.

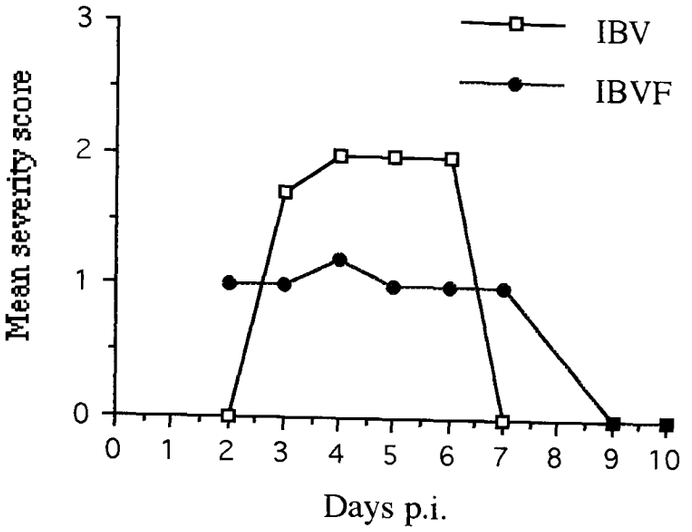


**Figure 10.1**. Consistency of nasal exudate in normal (IBV) and heteropaenic chickens (IBVF) infected with IBV. Each point represents the mean severity score of the birds with nasal exudate.

Table 10.5 shows the virus titres in trachea, lung and kidneys of normal and heteropaenic chickens infected with IBV. No significant differences in virus titres were recorded in these tissue samples on any of the days tested. No virus was recovered from untreated or 5 FU-treated control chickens.

**Table 10.5. Virus titres in selected tissues of IBV infected normal and heteropaenic chickens.**

| Tissues | Group | Days post-infection | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 3 | 5 | 7 | 10 |
| Trachea | IBV | 3.17 ± 0.58* | 3.50 ± 1.0 | 3.30 ± 0.72 | 2.50 ± 0.25 | < 2.0 |
| | IBVF | 2.83 ± 0.63 | 3.25 ± 1.3 | 4.00 ± 0.43 | 2.25 ± 0 | < 2.0 |
| Lung | IBV | < 2.0 | < 2.0 | 2.42 ± 0.14 | 2.58 ± 0.14 | < 2.0 |
| | IBVF | < 2.0 | < 2.0 | 2.83 ± 0.58 | 2.67 ± 0.14 | < 2.0 |
| Kidney | IBV | < 2.0 | < 2.0 | 2.58 ± 0.14 | 2.42 ± 0.14 | < 2.0 |
| | IBVF | < 2.0 | < 2.0 | 2.75 ± 0.43 | 3.00 ± 0.66 | < 2.0 |

* indicates mean (± s.d.) of virus titres expressed as $\log_{10}$ $CD_{50}$ per g, from three birds sampled on each occasion.

Table 10.6 shows the histopathological lesion scores in the tracheas of heteropaenic and normal chickens infected with IBV. Compared to normal chickens infected with IBV, epithelial degeneration and heterophilic infiltration were less in the heteropaenic chicks. No major differences in the lesion scores were seen with loss in ciliary activity and lymphocytic infiltration.

**Table 10.6. Severity of histopathological lesions in the trachea of IBV-infected normal and heteropaenic chickens.**

| Type of lesion | Groups | Days post-infection | | | |
|---|---|---|---|---|---|
| | | 3 | 5 | 7 | 10 |
| Cilia loss | IBV | 2.7* | 3.0 | 3.0 | 2.7 |
| | IBVF | 2.0 | 3.0 | 2.7 | 1.7 |
| Epithelial degeneration | IBV | 2.0 | 1.7 | 1.3 | 0.7 |
| | IBVF | 0.3 | 0 | 0.3 | 0.3 |
| Heterophil infiltration | IBV | 1.7 | 2.0 | 0.3 | 0 |
| | IBVF | 0.3 | 0.3 | 0 | 0 |
| Lymphocyte infiltration | IBV | 0 | 2.7 | 2.3 | 2.7 |
| | IBVF | 0 | 2.0 | 1.7 | 1.7 |

* : The values indicate the severity of lesions scored on a 0 to 3 scale. Each value represents a mean score determined from three sections examined.

IBV antigen was demonstrated by IP staining in the tracheal sections of all the three chickens tested in each of the IBV-infected group and the IBV-infected and 5 FU-treated group, on days 3, 5 and 7 p.i. No virus antigen was detected on day 10 p.i. in the infected groups or on any of the days in the control groups.

Table 10.7 shows the changes in the serum levels of lysozyme and acid phosphatase following IBV infection. Lysozyme levels were significantly higher in the infected chickens from day 1 p.i. to day 10 p.i. while no significant differences were observed in the levels of acid phosphatase on any of the days tested.

**Table 10.7. Levels of lysozyme and acid phosphatase in the serum of normal and IBV infected chickens (n = 5).**

| Days post-infection | Lysozyme (μg/ml) | | Acid phosphatase (U/lit) | |
|---|---|---|---|---|
| | Control | IBV infected | Control | IBV infected |
| 0 | 3.62 ± 0.5 | 3.62 ± 0.5 | 11.7 ± 2.0 | 11.7 ± 2.0 |
| 1 | 3.38 ± 0.3 | 3.98 ± 0.2* | 8.8 ± 1.4 | 10.8 ± 2.0 |
| 3 | 3.62 ± 0.3 | 4.22 ± 0.2* | 10.6 ± 2.0 | 10.6 ± 1.3 |
| 5 | 3.18 ± 0.2 | 4.34 ± 0.9* | 10.9 ± 1.5 | 9.4 ± 1.5 |
| 7 | 3.35 ± 0.3 | 4.26 ± 0.1* | 9.0 ± 1.4 | 9.9 ± 1.6 |
| 10 | 3.30 ± 0.3 | 4.18 ± 0.2* | 8.7 ± 1.5 | 10.6 ± 2.3 |

* indicates significant differences in comparison to control values.

TNLF activity was recorded in the serum of three of five IBV-infected chickens on day 10 p.i. only. Their percent cytotoxicity values were 31.4, 33.3 and 5.9. None of the other samples had any cytotoxic effect on the target cells.

## 10.4. DISCUSSION

Two drugs, cyclophosphamide and 5 FU have been used for *in-vivo* depletion of heterophils in chickens (Fulton et al., 1996; Kogut et al., 1993). Cyclophosphamide given at a dose of 75 mg/kg body-weight once daily for 4 consecutive days causes heteropaenia on days 10 to 12 after the last injection. Although this drug has been shown to cause a reversible heteropaenia, it has been conventionally used to abrogate B-cells, to study the pathogenesis of various diseases in chickens and turkeys (Chandra, 1988; Chubb et al., 1988; Jones et al., 1992). In contrast, a single intravenous injection of 5 FU has been shown to cause a selective reduction in the heterophil counts for a longer duration (Kogut et al., 1993). So, for the present study, this drug was chosen for induction of heteropaenia. Following a single injection of 5 FU, significant reductions in heterophil numbers were seen from 3 to 10 days p.t. followed by recovery to normal levels by day 13 p.t. These results were similar to those reported by Kogut et al.(1993). However, we found a transient lymphopaenia in the 5 FU treated chickens on days 3 and 6 p.t., unlike Kogut et al. (1993) who found no lymphopaenia in any of the treated birds. The reason for this discrepency is not clear.

Three days after 5 FU treatment, when the heterophil numbers were significantly lower, chickens were infected with IBV. Following infection, the numbers of birds

exhibiting clinical signs (nasal exudate) were significantly higher in the 5 FU-treated group. This may be attributed to the transient lymphocytopaenia seen following the drug treatment. It has been shown that abrogation of B-cells by surgical bursectomy, in an inbred line of chicken resistant to IBV (line C) produced more severe respiratory signs (Cook et al., 1991a). Depletion of T-cells by CSP treatment of chickens also caused more severe clinical signs following infection with IBV (Chapter 9). However, the nasal exudate in the heteropaenic chickens was characteristically copious, thin and watery enabling it to be easily squeezed out of the nasal cavity. It is possible that this could have also contributed to the increased numbers of birds with nasal exudate in the 5 FU-treated group. It has been shown that the heterophils are the most prevalent inflammatory cells recovered in the respiratory tract lavage of normal SPF chickens and their numbers increased dramatically in IBV infected chickens (Fulton et al., 1990; Fulton et al., 1993). The reduced numbers of heterophils in the 5 FU treated chickens is likely to explain the watery consistency of the nasal exudate.

No differences in virus titres were seen in trachea, lung or kidneys of normal and heteropaenic chickens infected with IBV. The presence of IBV antigen, as detected by immunochemistry, in the tracheas of the two groups of birds was also similar. These results suggest that heterophils do not play an important role in IBV clearance. *In-vitro* studies with chicken heterophil peptides have also shown that, though they were bactericidal and fungicidal, they were unable to neutralise IBV (Evans et al., 1995).

In the heteropaenic chickens infected with IBV, the severity of epithelial cell damage was found to be less than in normal infected chickens. Several workers have found that in neutropaenic animal models, when neutrophils are prevented from being involved in the inflammatory response, the damaging effect to the host is reduced (Slocombe et al., 1985; Breider et al., 1988).

The enzymes of the oxygen-independent mechanisms like lysozyme and acid phosphatase are responsible for microorganism digestion and are released into the surrounding environment when they are unable to engulf organisms or when inflammatory cells are lysed during an inflammatory response (Fulton et al., 1996). Following infection of normal chickens with IBV, although lysozyme levels were significantly higher from days 1 to 10 p.i. no changes in acid phosphatase levels were seen.

For the assay of TNLF activity a murine cell line sensitive to TNF was used. Although murine cells have been used to assay chicken TNLF activity in spleen cell supernatants (Byrnes et al., 1993) it has been shown that chicken fibroblast cell lines are more

sensitive (Zhang and Lillehoj, 1995). Though detectable levels of TNLF were found in 60% of the birds on day 10 p.i., it can be argued that serum samples from infected chickens may contain other toxic factors that could kill target cells. It has been shown that interleukin 1, interleukin 6 and interferons can cause cytotoxicity in some tumour cell types. Products of complement activation and other cascades involved in inflammation and coagulation have the potential for tumour cell killing (Kurzman et al., 1993). Hence, more comphrehensive studies are required to ascertain the role of TNLF in IBV infections.

In summary, it has been shown that heterophils are ineffective in neutralisation of IBV and in fact contribute to the severity of lesions (probably by release of lysozyme) seen in the tracheal epithelium.

# CHAPTER 11

## CROSS-REACTIVE CELLULAR IMMUNE RESPONSES IN CHICKENS VACCINATED WITH LIVE INFECTIOUS BRONCHITIS VIRUS VACCINE

**CONTENTS** **PAGE NUMBER**

# CHAPTER 11

# CROSS-REACTIVE CELLULAR IMMUNE RESPONSES IN CHICKENS VACCINATED WITH LIVE INFECTIOUS BRONCHITIS VIRUS VACCINE

## 11.1 INTRODUCTION

Infectious bronchitis virus infects the respiratory tract, kidneys and oviduct of chicks of all ages causing retarded growth, mortality, reduced egg production and inferior egg shell quality (King and Cavanagh, 1991). The virion consists of three structural proteins, the spike glycoprotein (S), comprising two subunits S1 and S2, protruding from the viral envelope, the integral membrane glycoprotein (M) and the nucleocapsid protein (N) associated with the viral ribonucleic acid.

IBV strains have been classified into several serotypes based on VN tests. It has been observed that IBV vaccines not only protect chickens against challenge with the same serotype but also induce varying degrees of cross-protection against strains of other serological types (Cook et al., 1986). Cross-immunity tests in experimental chickens with different IBV strains have shown different levels of reciprocal or unidirectional cross-immunity which did not reflect the results of *in-vitro* VN tests (Raggi and Lee, 1965; Winterfield and Fadly, 1972; Darbyshire, 1985; Arvidson et al., 1990). This discrepancy is due to the fact that serotype-specific VN antibodies are induced mainly by the S1 subunit of the S glycoprotein (Cavanagh et al., 1986; Kant et al., 1992) while antibodies to either the conserved sequences on the S1 protein or other less-variable proteins may also play a role in protective immunity of the chicken. Although the S1 protein has been regarded as the most likely inducer of protection (Cavanagh et al., 1986; Ignjatovic and Galli, 1994), Ignjatovic and Galli (1995) have suggested that the S2 and N proteins might also be of importance in IBV immunity since they carried epitopes for induction of cross-reactive antibodies.

Cell mediated immune (CMI) responses have been suggested to play an important role in immunity to IBV (Chubb, 1974; Darbyshire and Peters, 1984; Cook et al., 1992) and have been demonstrated in IBV-infected chickens (Timms and Bracewell, 1981; Chubb et al., 1988; Janse et al., 1994; Wakenell et al., 1995; see also Chapter 8). Although immunisation with N or M proteins did not result in protective immunity (Ignjatovic and Galli, 1994), Boots et al. (1992) have demonstrated that N protein contains T-cell epitopes involved in protection. Subsequently, Ignjatovic and Galli

(1995) have shown that purified S1, S2, N and M proteins of the N1/62 IBV isolate induced CMI responses detected by delayed hypersensitivity reactions. Together, these findings show that all the IBV proteins induce both humoral and cellular immune responses. However, although the cross-reactivity of humoral antibodies is known (Ignjatovic and Galli, 1995), the cross-reactivity of CMI responses has not been studied.

An early response of activated T-cells when appropriately triggered by mitogen or antigen is to release lymphokines. Interleukin-2 (IL-2) and gamma-interferon (IFN-γ) are among the most important lymphokines; IL-2 interacts with the cells of the immune system to influence their differentiation and effector functions, including the secretion of other lymphokines while IFN-γ regulates several immunological reactions such as the activation of macrophages and enhancement of immunogenicity through expression of major histocompatibility complex antigens.

The aim of the present study was to establish whether cross-protection seen in IBV vaccinated chickens is also as a result of cross-reactive cellular immune responses stimulated by the vaccine virus to serologically distinct strains of IBV.

## 11.2 Materials and Methods

### 11.2.1 Chickens

Embryonated SPF hen's eggs were obtained from a commercial source and hatched in our laboratory as described in Chapter 3. The chicks were maintained in flexible isolators and fed and watered *ad-libitum*.

### 11.2.2 Viruses

A commercial H120 vaccine was passaged via the allantoic cavity of 10-day old embryonated SPF eggs and the allantoic fluid collected 48 hrs after inoculation was used to vaccinate chickens at two weeks of age.

After partial purification and inactivation, three serologically distinct virulent viruses, IBV strains M41 (Ambali and Jones, 1991a), 7 (Cook et al., 1986) and 793/B-like (Chapter 8) were used for *in-vitro* stimulation of spleen mononuclear cells (MNC).

### 11.2.3 Preparation of partially purified and inactivated viruses

The three virulent IBVs were propagated in 10-day old embryonated SPF eggs and the allantoic fluids were collected 48 hrs after infection. These were centrifuged at 3000g for 30 min and the virus pelleted at 48,000g for 1 hr in an ultracentrifuge. The virus pellet was resuspended in PBS pH 7.2 to 1 in 30 of its original volume. Virus titres were determined in TOC and the 3 viruses were diluted to contain 7.0 $\log_{10}$ $CD_{50}$ per ml. They were inactivated by incubation with 0.05% ß-propiolactone (Sigma) at $37^0$C for 4 hours and then overnight at $4^0$C (Ignjatovic and Galli, 1995). Absence of residual infectivity in the preparation of inactivated virus was confirmed by titration in TOC. Allantoic fluids collected from uninfected eggs were processed similarly and used as control antigen.

### 11.2.4 Preparation of spleen MNC

Spleens were removed aseptically from chickens post-mortem and each spleen was handled separately. The capsules were removed and the tissue was gently forced through a 60 µm-mesh (Sigma) using the plunger from a 5-ml syringe. The cell suspension was recovered in 4 ml of RPMI 1640 medium (Life Technologies, Paisley, Scotland) containing 200 IU of penicillin and 200 µg streptomycin per ml (serum-free RPMI) and layered on to 3 ml of lymphocyte separation medium (Life Technologies). After centrifugation at 1000 g for 20 minutes, MNC were recovered from the interface and washed twice in serum-free medium and the number of viable cells determined by the trypan blue exclusion technique. The cells were resuspended to 1 x $10^7$/ml in RPMI containing 10% foetal-calf serum (Life Technologies), 1mM sodium pyruvate, 1mM non-essential amino acids and 5 x $10^{-5}$ M 2-mercaptoethanol (complete RPMI).

### 11.2.5 Antigen-specific lymphoproliferation

Antigen stimulation was carried out by incubating 100µl of spleen cell suspensions with 50 µl of appropriately diluted inactivated virus antigens or 10µg/ml final concentration of Con A in 96-well microplates for 4 days at $37.5^0$C in a humidified atmosphere of 5% $CO_2$. Eighteen hours before harvesting 0.5 µCi of tritiated thymidine ($^3$H) (Amersham) was added to each well. Cells were harvested using a cell-harvester (Skatron) and the incorporated radioactivity was measured by liquid scintillation counting (Chapter 7). The background $^3$H uptake was measured using control antigen or medium in place of viral antigen and Con A. The assay was performed in triplicate and the results were expressed as cpm values.

### 11.2.6 Lymphokine assays

For lymphokine assays, 100 µl of cells were incubated with 100 µl of antigen or Con A (10µg/ml) in 48-well plates for 3 hours followed by the addition of 300 µl of complete medium. Supernatants were collected from these cultures after 48 hrs incubation and stored frozen at -70 $^0$C until used for IL-2 and IFN-γ assays.

#### 11.2.6.1 IL-2 assay

The method of assay for IL-2 was similar to that described by Adair et al. (1991) and Isobe and Lillehoj (1993), using Con A-stimulated T-lymphoblasts as assay cells. Spleen MNC suspensions prepared from 5-week-old SPF chickens were stimulated with 10 µg/ml Con A for 48 hrs. Viable T-lymphoblast cells were isolated on lymphocyte seperation medium, treated with 0.05M methyl-∞-D-mannopyranoside (Sigma), adjusted to 2 x $10^6$ cells/ml in complete RPMI and cultured with the supernatants to be tested for 2 days. The proliferation of T-lymphoblast cells was determined by the addition of 0.5 µCi of $^3$H thymidine per well. Eighteen hours later, the cells were harvested onto filtermats, dried and the incorporated radioactivity counted. The results were expressed as cpm. Supernatants collected from normal spleen MNC stimulated with 10 µg/ml Con A were used as a positive control. These samples (n =7) had a mean (± s.d) cpm value of 8074 (± 1048).

#### 11.2.6.2 Interferon assay

IFN-γ activity was assessed using a viral-inhibition assay as described by Prowse and Pallister (1989) and Adair et al. (1991). Primary cultures of chicken embryo fibroblast (CEF) prepared from 10-day old SPF eggs were trypsinised, centrifuged and resuspended in M199 medium (Life Technologies) containing tryptose phosphate broth, HEPES and 5% calf serum (see Appendix). Cells were counted and the concentrations adjusted to 0.5 x $10^6$ per ml. One hundred microlitre volumes of cells were added to the wells of flat-bottom microtitre plates and the plates were incubated at 37$^0$C in 5% $CO_2$ for 24 hrs. After monolayers developed, the medium was removed and replaced with doubling dilutions of supernatants to be tested. After 18 hrs the supernatants were removed and replaced with 100 µl amounts of Semliki Forest Virus (kindly supplied by Dr. Adair, Veterinary Sciences Division, Stormont, Belfast) containing 100 tissue culture infective doses ($TCID_{50}$) per well. After 48 hours the CEF viability was measured using the MTT method described in Chapter 10. Briefly, 20 µl of 5 mg/ml solution of MTT in PBS was added to each well and the plates were incubated at 37$^0$C for 6 hrs. Then, 100 µl of extraction buffer (12.5% sodium dodecyl sulphate in 50% dimethyl formamide, pH 4.7) was added. Following overnight incubation at 37$^0$C, the absorbance was read at 570 nm in an ELISA reader (Dynatech Laboratories, UK). The interferon titre was taken as the dilution of supernatants showing complete protection of

CEF viability. The results were expressed as mean $log_2$ IFN-γ titres from duplicate determinations.

Each assay included as positive control, a supernatant from 10 μg/ml Con A stimulated cells collected after 24 hrs activation. In six different assays, this control had a mean (± s.d) $log_2$ titre of 9.7 (± 0.52).

### 11.2.7 Tracheal organ cultures

TOC prepared from 19- or 20-day old embryonated SPF chicken eggs following the method described in Chapter 3 were used for virus titrations. Virus titres were expressed as median ciliostatic doses ($CD_{50}$).

### 11.2.8 Virus neutralisation tests

VN tests were performed in TOC using 100 $CD_{50}$ of homologous or heterologous virus against $log_2$ dilutions of chicken sera (Chapter 3).

### 11.2.9 Experimental Design

Two-week old SPF chickens were vaccinated with 5.0 $log_{10}$ $CD_{50}$ of H120 vaccine virus intra-nasally. On days 4, 7, 11 and 14 post-vaccination (p.v.), four vaccinated chickens and four age-matched controls were killed and their spleens collected. Spleen MNC were prepared and used for antigen or Con A (10 μg/ml)-induced lympho-proliferation assays. Supernatants for IL-2 and IFN-γ assays were collected from antigen or mitogen-stimulated spleen MNC cultures as described in Section 11.2.6. Spleen MNC collected from control or H120 vaccinated chickens were stimulated with virus-antigen prepared from homologous (M41) and heterologous (7 and 793/B-like) viruses.

Tracheas were collected from these birds on days 4, 7, 11 and 14 p.v. for virus titrations. Sera prepared from these chickens on day 14 p.v. were used for assessment of VN antibodies.

### 11.2.10 Statistical analysis

The cpm values indicating levels of IL-2 and $log_2$ IFN-γ titres were compared by one-way analysis of variance and Fischer's least significant difference tests. A p value of less than 0.05 was considered significant.

## 11.3 RESULTS

No antigen-specific lymphoproliferative responses either to homologous or heterologous antigens were seen in spleen MNC collected from vaccinated chickens on days 4 and 7 p.v On days 11 and 14 p.v., 4 / 8 chickens responded to the homologous antigen (strain M41) and 1 / 8 to the heterologous antigen (strain 7) and none to strain 793/B (Table 11.1). Although the cpm values were very low, the SI (cpm of antigen-stimulated cultures/cpm of control antigen cultures) varied from 5.2 to 12.2.

The vitality of spleen MNC from each chicken could be shown by their responses to Con A (Table 11.2). The proliferative response to Con A of spleen MNC from vaccinated chickens was significantly reduced on day 7 p.v.

**Table 11.1. Proliferative responses of spleen MNC from control and H120-vaccinated chickens stimulated with M41, 7 and 793/B antigens.**

| Group/ days p.v. | M41 | | 7 | | 793/B | | Control antigen | |
|---|---|---|---|---|---|---|---|---|
| | 11 | 14 | 11 | 14 | 11 | 14 | 11 | 14 |
| Control | 0.09 | 0.04 | 0.05 | 0.06 | 0.09 | 0.06 | 0.06 | 0.04 |
| | 0.06 | 0.09 | 0.04 | 0.07 | 0.06 | 0.09 | 0.06 | 0.07 |
| | 0.07 | 0.07 | 0.06 | 0.06 | 0.06 | 0.06 | 0.07 | 0.05 |
| | 0.09 | 0.09 | 0.06 | 0.08 | 0.06 | 0.06 | 0.06 | 0.05 |
| H120 vaccine | **0.30** | **0.52** | 0.09 | **0.31** | 0.13 | 0.10 | 0.04 | 0.06 |
| | 0.05 | **0.61** | 0.09 | 0.20 | 0.09 | 0.09 | 0.09 | 0.05 |
| | 0.08 | **0.42** | 0.07 | 0.11 | 0.09 | 0.08 | 0.09 | 0.06 |
| | 0.07 | 0.10 | 0.04 | 0.09 | 0.04 | 0.09 | 0.08 | 0.04 |

Results are expressed as cpm x $10^3$. Values in bold were regarded positive.

**Table 11.2. Proliferative responses of spleen MNC to Con A. Results expressed in cpm (x $10^3$) (mean ± S.D.).**

| Source of spleen cells | Mitogen | Days post-vaccination | | | |
|---|---|---|---|---|---|
| | | 4 | 7 | 11 | 14 |
| control chickens | Con A | 40.6 ± 6.57[a] | 71.26 ± 9.15[a] | 68.51 ± 48.79[a] | 117.83 ± 37.57[a] |
| control chickens | medium | 4.45 ± 3.21[b] | 2.26 ± 1.02[b] | 1.7 ± 0.66[b] | 2.76 ± 1.38[b] |
| H120 vaccinated chickens | Con A | 30.42 ± 15.56[a] | 40.63 ± 21.68[c] | 52.54 ± 24.65[a] | 108.64 ± 41.33[a] |
| H120 vaccinated chickens | medium | 4.14 ± 0.35[b] | 2.12 ± 0.44[b] | 2.47 ± 0.88[b] | 3.26 ± 1.09[b] |

Values between groups with different superscripts differ significantly.

Tables 11.3, 11.4 and 11.5 show the mean cpm values indicating the levels of IL-2 production in the supernatants of control or virus-antigen stimulated spleen MNC from control or vaccinated chickens. With the homologous antigen, strain M41 (Table 11.3), the IL-2 levels of control chickens stimulated with the antigen, were significantly higher than those of control or vaccinated chickens stimulated with the control antigen on all days tested. The IL-2 levels in vaccinated chickens stimulated with the virus-antigen were significantly higher than those of other three combinations on all days tested. Within the group of vaccinated chickens stimulated with IBV strain M41, IL-2 levels on day 11 were higher than those on days 4, 7 and 14 p.v. and the levels on day 7 p.v. was higher than those on day 4 p.v. No differences in IL-2 levels were seen within other groups.

**Table 11.3. Production of IL-2 in the supernatants of spleen cells stimulated with strain M41. Results expressed in cpm (mean ± S.D.).**

| Source of spleen cells | Antigen | Days post-vaccination | | | |
|---|---|---|---|---|---|
| | | 4 | 7 | 11 | 14 |
| control chickens | M41 | 779.8 ± 143.1[a] | 709.0 ± 243.3[a] | 668.0 ± 53.7[a] | 803.5 ± 97.9[a] |
| control chickens | control antigen | 342.5 ± 63.4[b] | 326.0 ± 50.3[b] | 375.3 ± 25.4[b] | 360.0 ± 51.1[b] |
| H120 vaccinated chickens | M41 | 1279.3 ± 142.7[c] | 1488.5 ± 25.9[c] | 1786.8 ± 193.1[c] | 1375.3 ± 89.1[c] |
| H120 vaccinated chickens | control antigen | 486.8 ± 227.0[b] | 301.0 ± 35.7[b] | 384.8 ± 32.2[b] | 392.0 ± 24.6[b] |

Values between groups with different superscripts differ significantly.

Spleen MNC from control chickens stimulated with strain 7 (Table 11.4), didnot produce IL-2 in an amount significantly different from the control or vaccinated chickens stimulated with the control antigen. However, the MNC of vaccinated chickens stimulated with IBV strain 7 produced levels of IL-2 significantly different from other three combinations on days 7, 11 and 14 p.v. Within the group of vaccinated chickens stimulated with strain 7, the level of IL-2 on day 11 was significantly higher than that on day 4 (Table 11.4). Similar results were seen with spleen MNC stimulated with IBV strain 793/B-like (Table 11.5). However, no differences were seen within any group stimulated with this strain.

**Table 11.4. Production of IL-2 in the supernatants of spleen cells stimulated with strain 7.**

| Source of spleen cells | Antigen | Days post-vaccination | | | |
|---|---|---|---|---|---|
| | | 4 | 7 | 11 | 14 |
| control chickens | 7 | 523.5 ± 240.8 | 457.0 ± 211.7$^{ab}$ | 653.3 ± 227.2$^{a}$ | 553.3 ± 227.9$^{a}$ |
| control chickens | control antigen | 342.5 ± 63.4$^{a}$ | 326.0 ± 50.3$^{b}$ | 375.3 ± 25.4$^{a}$ | 360.0 ± 51.1$^{a}$ |
| H120 vaccinated chickens | 7 | 795.8 ± 193.0$^{b}$ | 946.5 ± 350.4$^{c}$ | 1292.5 ± 448.7$^{b}$ | 1033.5 ± 193.6$^{b}$ |
| H120 vaccinated chickens | control antigen | 486.8 ± 227.0 | 301.0 ± 35.7$^{b}$ | 384.8 ± 32.2$^{a}$ | 392.0 ± 24.6$^{a}$ |

Values between groups with different superscripts differ significantly.

**Table 11.5. Production of IL-2 in the supernatants of spleen cells stimulated with strain 793/B-like.**

| Source of spleen cells | Antigen | Days post-vaccination | | | |
|---|---|---|---|---|---|
| | | 4 | 7 | 11 | 14 |
| control chickens | 793/B-like | 460.3 ± 127.9 | 542.0 ± 163.2$^{a}$ | 439.5 ± 83.5$^{a}$ | 574.3 ± 335.6$^{a}$ |
| control chickens | control antigen | 342.5 ± 63.4$^{a}$ | 326.0 ± 50.3$^{a}$ | 375.3 ± 25.4$^{a}$ | 360.0 ± 51.1$^{a}$ |
| H120 vaccinated chickens | 793/B-like | 743.0 ±212.9$^{b}$ | 782.3 ± 229.7$^{b}$ | 871.0 ±183.4$^{b}$ | 985.8 ±316.7$^{b}$ |
| H120 vaccinated chickens | control antigen | 486.8 ± 227.0 | 301.0 ± 35.7$^{a}$ | 384.8 ± 32.2$^{a}$ | 392.0 ± 24.6$^{a}$ |

Values between groups with different superscripts differ significantly

When the IL-2 levels in the supernatants of virus-antigen stimulated cultures from vaccinated chickens on day 11 p.v. were compared, the highest levels were seen in IBV strain M41 stimulated cultures, followed by IBV strain 7 and then 793/B-like stimulated cultures.

The levels of IL-2 in supernatants of Con A stimulated MNC from vaccinated and control chickens are shown in Table 11.6. Compared to unvaccinated controls, significant reduction of IL-2 production in vaccinated chickens was seen on day 7 p.v.

**Table 11.6. Production of IL-2 in the supernatants of spleen cells stimulated with Con A.**

| Source of spleen cells | Mitogen | Days post-vaccination | | | |
|---|---|---|---|---|---|
| | | 4 | 7 | 11 | 14 |
| control chickens | Con A | 4571 ± 1452[a] | 7960 ± 1327[a] | 7755 ± 745[a] | 10908 ± 1749[a] |
| control chickens | medium | 796 ± 193[b] | 875 ± 270[b] | 982 ± 298[b] | 1347 ± 631[b] |
| H120 vaccinated chickens | Con A | 3873 ± 2215[a] | 4029 ± 1128[c] | 7060 ± 1885[a] | 9148 ± 2095[a] |
| H120 vaccinated chickens | medium | 983 ± 298[b] | 798 ± 478[b] | 671 ± 163[b] | 1056 ± 600[b] |

Values between groups with different superscripts differ significantly

IFN-γ assay results are presented in Tables 11.7, 11.8 and 11.9. Very little background levels of IFN-γ was seen in supernatants of spleen MNC from control or vaccinated chickens stimulated with the control antigen. Levels of IFN-γ in vaccinated chickens stimulated with strain M41 were significantly lower than controls on day 7 p.v. and higher on day 14 p.v.(Table 11.7). With strain 7, the IFN-γ levels in vaccinated chickens were lower than those of control birds on days 4 and 7 p.v. but higher on days 11 and 14 p.v. (Table 11.8). With strain 793/B, IFN-γ levels in vaccinated chickens were lower than those of control birds on day 7 p.v. and never higher (Table 11.9). No differences were seen in IFN-γ levels in supernatants of Con A stimulated MNC from control or vaccinated chickens.

**Table 11.7. Production of IFN-γ in the supernatants of spleen cells stimulated with strain M41. Results expressed as $\log_2$ IFN-γ titres.**

| Source of spleen cells | Antigen | Days post-vaccination | | | |
|---|---|---|---|---|---|
| | | 4 | 7 | 11 | 14 |
| control chickens | M41 | 5.0 ± 1.83[a] | 6.0 ± 0[a] | 4.75 ± 0.5[a] | 4.25 ± 0.5[a] |
| control chickens | control antigen | 0[b] | 0[b] | 1.75 ± 1.23[b] | 0[b] |
| H120 vaccinated chickens | M41 | 4.75 ± 0.96[a] | 4.25 ± 2.06[c] | 5.75 ± 1.89[a] | 6.25 ± 2.22[c] |
| H120 vaccinated chickens | control antigen | 0[b] | 0[b] | 0[b] | 0[b] |

Values between groups with different superscripts differ significantly

**Table 11.8. Production of IFN-γ in the supernatants of spleen cells stimulated with strain 7. Results expressed as $log_2$ IFN-γ titres.**

| Source of spleen cells | Antigen | Days post-vaccination | | | |
|---|---|---|---|---|---|
| | | 4 | 7 | 11 | 14 |
| control chickens | 7 | $5.0 \pm 0.82^a$ | $4.75 \pm 0.96^a$ | $4.00 \pm 0.82^a$ | $3.25 \pm 0.96^a$ |
| control chickens | control antigen | $0^b$ | $0^b$ | $1.75 \pm 1.23^b$ | $0^b$ |
| H120 vaccinated chickens | 7 | $3.5 \pm 0.58^c$ | $2.25 \pm 0.96^c$ | $6.25 \pm 0.96^c$ | $6.00 \pm 2.16^c$ |
| H120 vaccinated chickens | control antigen | $0^b$ | $0^b$ | $0^d$ | $0^b$ |

Values between groups with different superscripts differ significantly

**Table 11.9. Production of IFN-γ in the supernatants of spleen cells stimulated with strain 793/B-like. Results expressed as $log_2$ IFN-γ titres.**

| Source of spleen cells | Antigen | Days post-vaccination | | | |
|---|---|---|---|---|---|
| | | 4 | 7 | 11 | 14 |
| control chickens | 793/B | $3.75 \pm 0.96^a$ | $3.25 \pm 1.23^a$ | $5.5 \pm 0.58^a$ | $3.25 \pm 0.5^a$ |
| control chickens | control antigen | $0^b$ | $0^b$ | $1.75 \pm 1.23^b$ | $0^b$ |
| H120 vaccinated chickens | 793/B | $1.5 \pm 1.73^b$ | $1.5 \pm 1.0^c$ | $5.75 \pm 2.63^a$ | $3.25 \pm 0.5^a$ |
| H120 vaccinated chickens | control antigen | $0^b$ | $0^b$ | $0^b$ | $0^b$ |

Values between groups with different superscripts differ significantly

The mean virus titres in the trachea of vaccinated chickens were $3.75 \pm 0.5$, $4.3 \pm 0.38$, $2.5 \pm 0$ and $<2.0$ $log_{10}$ $CD_{50}$ on days 4, 7, 11 and 14 p.v. respectively. On day 14 p.v., the $log_2$ VN titre in the sera of vaccinated chickens tested with homologous antigen (strain M41) was $4.0 \pm 0$. The titres with heterologous antigens (strains 7 and 793/B-like) were less than $log_2$ 3.0.

## 11.4 DISCUSSION

Following vaccination of chickens with a live attenuated IBV vaccine, the presence of cross-reactive cellular immune responses has been shown. These responses varied in magnitude with the serotype of IBV strain used for *in-vitro* stimulation.

IBV strain M41 is the virulent virus from which the H120 vaccine has been prepared by 120 serial passages in embryonated chicken eggs. Strain 7, is a non-Massachusetts variant isolated from the UK (Cook, 1984). Using the challenge model developed by Smith et al. (1985), the H120 vaccine has been shown to partially protect chickens against this strain (Cook et al., 1986). The mortality in vaccinated chickens against challenge with this strain along with pathogenic strains of *E.coli* was 5% against 14% in unvaccinated chickens (Cook et al., 1986). The H120 vaccine has been shown to afford little protection to the recent variant strain isolated from the UK and variably designated as 4/91 (Parsons et al., 1992) or 793/B (Gough et al., 1992), using protection of tracheal ciliary activity as a measure of immunity. Thus, the three IBV strains chosen for *in-vitro* stimulation of spleen MNC prepared from H120 vaccinated chickens, represents one that was homologous to the vaccine virus (strain M41), one against which the vaccine offered partial protection (strain 7) and one against which the vaccine was ineffective (strain 793/B-like).

Using these three strains of IBV, the hypothesis tested in this work was that partial protection afforded by the H120 vaccine to the heterologous virus could be attributed to cross-reactive cellular immune responses induced by vaccine virus. Antigen-specific lymphoproliferation and antigen-induced IL-2 and IFN-γ were used as a measure of cellular immunity.

Antigen-specific lymphoproliferation was induced in only 1/8 chickens by the serologically distinct strain 7, while 4/8 birds showed responses to the homologous antigen, IBV M41. Timms and Bracewell (1981) have also shown that antigen-specific lymphoproliferation in H120 vaccinated chickens were seen from day 5 p.v. Boots (1991) also found antigen-specific stimulation in 2 of 8 chickens at 2 weeks post-primary infection with M41. In the present study, the cpm of control or virus antigen-stimulated cultures were very low. The cpm reported by Boots (1991) using virulent M41 strain was about twice that reported here, but Timms and Bracewell (1981) expressed results as stimulation indices. This low response was not due to lack of vitality of these cells since they showed high proliferative responses to Con A. The mitogen-induced lymphoproliferative responses and IL-2 production were lower in the

vaccinated chickens than unvaccinated ones on day 7 p.v., when the virus titres in the trachea were the highest.

A surprising finding was that all three viruses induced IL-2 production from MNC of control chickens. Although only M41 induced levels which were significant, this phenomenon deserves further study. It is known that some viruses like rabies virus act as 'superantigens' and trigger a powerful T-cell response (Herman et al., 1991). This could result in IBV-specific tolerance and may be a mechanism by which IBV can avoid immunological surveillance.

However, all the three strains of IBV had stimulated the production of IL-2 in the supernatants of MNC from H120-vaccinated chickens, with peak levels on day 11 p.v. Whether these levels were related to protection is not known.

The role of IFN-γ in IBV infections is conflicting. Otsuki et al. (1988) detected variable titres of IFN-γ in chickens with various strains of IBV whereas other workers (Lomniczi, 1974; Holmes and Darbyshire, 1978) could not detect IFN-γ in serum or organ cultures of chickens infected with IBV. Although the results of IFN-γ assay in this study were variable, the heterologous strain 7 stimulated the production of IFN-γ on days 11 and 14 p.v. but strain 793/B never did.

Taken together, these results show that cellular immune responses induced by a live IBV vaccine are cross-reactive and do not reflect the results of *in-vitro* serological differentiation of IBV strains by the VN test. This lack of serotype-specificity in cellular immune responses to IBV could have contributed to variable cross-protection seen in *in-vivo* studies (Cook et al., 1986). Although the amino acid sequences of the S protein differ greatly between serotypes and indeed of strains within the same serotype (Kusters et al., 1990), the N and M proteins are less variable (Williams et al., 1992; Binns et al., 1986) and there are conserved sequences even within the S protein (Wang et al., 1995). These sequence identities among the proteins could have contributed to this cross-reactivity. This is the first evidence of cross-reactive cellular immune responses among IBV strains. Further studies are needed to identify and characterise cross-reactive epitopes among them.

# CHAPTER 12

## EFFECT OF STORAGE OF CHICKEN AND TURKEY BLOOD ON THE LYMPHOCYTE RESPONSES TO CONCANAVALIN A AND POKEWEED MITOGEN

**CONTENTS** **PAGE NUMBER**

# CHAPTER 12

## EFFECT OF STORAGE OF CHICKEN AND TURKEY BLOOD ON THE LYMPHOCYTE RESPONSES TO CONCANAVALIN A AND POKEWEED MITOGEN

### 12.1. INTRODUCTION

Lymphocyte proliferation in response to plant lectins is routinely used as a measure of cellular immunity in many animal species including chickens and turkeys. Three types of lectins, Con A, PHA and PWM have been used for stimulation of avian lymphocytes. PHA and Con A stimulate T-lymphocytes (Hovi et al.,1978 ; Toivanen and Toivanen, 1973) while PWM can stimulate both B and T-lymphocytes (Weber,1973; Vainio and Ratcliffe, 1984).

Whole blood assays are considered a more valid and a truer measure of cellular immune competence than those using purified lymphocytes (Lee, 1978). Since only small quantities of blood are required and large numbers of samples can be processed simultaneously, whole blood mitogenic assays are convenient for monitoring the functional capability of immune cells in chicken and turkey flocks. However, the applicability of this assay on a flock basis may be hampered due to individual variations seen between birds. This may be due to fluctuations in the number of responder cells present in the circulation at the time of blood collection or may reflect genetic diversity among birds (Sharma and Belzer,1992). It is known that the response of chicken T-cells to Con A is under genetic control (Miggiano et al., 1976). Most of the commercial chicken and turkey flocks originate from outbred breeder stocks.

Despite a certain degree of variation, it may still be possible to detect alterations in group responses following exposure to immunodepressive disease agents (Lee et al., 1978; Sharma and Lee, 1983; Cloud et al., 1992). This test is likely to be of value in the assessment of efficacy of vaccines in inducing cell-mediated immunity.

Critical parameters such as the choice of media, length and temperature of incubation and concentrations of mitogens for optimal *in-vitro* response of whole blood lymphocytes of chicken and turkeys have been well established (Lee,1978; Sharma and Belzer,1992; Talebi et al., 1995). However, the effect of storage of blood on the mitogen response has not been studied. This information may be vital if this test is to be applied to large numbers of birds

under experimental or field conditions. The objective of this study was to determine the effect of storing whole blood from chicken and turkeys on the lymphocyte responses to mitogens Con A and PWM.

## 12.2. MATERIALS AND METHODS

### 12.2.1 Chickens

#### 12.2.1.1. White leghorn chickens

SPF white leghorn (WLH) chicken eggs were obtained from a commercial source and hatched in our laboratory. The chicks were maintained under strict isolation conditions. Heparinised bloods collected from eight 3-4-week and five 5-week old chickens were used in mitogen assays.

#### 12.2.1.2. WLH layers in production

Heparinised blood collected from five 48-week old SPF WLH laying birds in production were used to determine mitogen responses to Con A.

#### 12.2.1.3. Broiler-type chickens

Day-old broiler chicks obtained from a commercial source were maintained in strict isolation in our laboratory. Heparinised blood was collected from six birds when they were 3- and 5-week old and used to assess mitogen responses to Con A.

### 12.2.2 Turkeys

Fertile turkey eggs were obtained from a commercial source, with no history of disease. The parent flocks were serologically tested weekly for mycoplasmas and periodically for TRTV and avian influenza virus and were found to be free of them. The eggs were hatched in our laboratory and poults were maintained under strict isolation conditions. Heparinised blood collected from ten 3 - 4-week old turkey poults were used in mitogen assays.

### 12.2.3 Mitogen assays

#### 12.2.3.1 Chicken blood

Mitogenic assays with chicken blood were performed following the procedure of Talebi et al. (1995) with slight modifications. Briefly, the heparinised blood was diluted 1 in 40 in RPMI 1640 medium containing L-glutamine and HEPES (Life Technologies, Paisley, Scotland) supplemented with 100 IU penicillin and 100μg streptomycin per ml. Volumes of 200 μl of the diluted blood were added to nine wells of 96-well flat-bottom tissue culture microplates. Three wells each received an additional 20μl of medium containing either Con A or PWM (Sigma) to yield a concentration of 2 μg per well. The remaining three wells received only medium without mitogens.

#### 12.2.3.2 Turkey blood

Mitogenic response of turkey blood cells were assessed following the methods of Sharma and Belzer (1992). Heparinised blood was diluted 1 in 20 in RPMI 1640 medium without HEPES (Sigma) but containing 7.5% normal turkey serum and antibiotics. Volumes of 100μl of this diluted blood were added to nine wells of a 96-well flat bottom microtitre plate. Three wells received 100 μl medium containing 32 μg of Con A, another set of three wells received 100 μl medium containing 8 μg of PWM. The remaining 3 wells received the same volume of medium without mitogens.

Both chicken and turkey blood cultures were incubated at 37.5$^{0}$C for 56 hours in a humidified atmosphere containing 5% $CO_2$. They were pulsed with 0.05μCi of $^{3}$H - thymidine (Amersham Life Sciences, Amersham, U.K.) for 16 hours prior to harvesting onto filter mats (Skatron Instruments, Suffolk, U.K.) using a cell harvester (Skatron, Herbyen, Norway). The discs were punched out and placed into disposable scintillation tubes and mixed with 2 ml of scintillation fluid (Optiphase 'Safe', Wallac UK, Milton Keynes, England). Radioactivity was measured in counts per minute (cpm) using a scintillation counter (Packard Tricarb, Berks, England). The stimulation index (S.I.) for each sample was calculated according to the following formula:

$$\text{S.I.} = \frac{\text{Mean cpm of stimulated cultures}}{\text{Mean cpm of unstimulated cultures}}$$

### 12.2.4 Experimental design

Cultures from diluted whole blood from chickens or turkeys were made within 30 minutes of collection. The S.I. values of these cultures were considered to be representative of 0 hours post-collection. Duplicate sets of blood were stored at $4^0$C and at room temperature (RT) ($22^0$C). After 24, 48 and 72 hours post-collection the stored whole blood was used in mitogenic assays.

Heparinised blood collected from 3 - 4-week old WLH chickens and turkeys was used to assess mitogen responses to Con A and PWM. For blood collected from other groups of birds only mitogen responses to Con A were determined.

### 12.2.5 Statistical analysis

The S.I. of stored blood was compared with that of unstored blood using students 't' test at 5% level of probability ($p < 0.05$). The mean percentage reduction in S.I. for blood stored for the first 24 hrs was calculated and compared by t-test ($p<0.05$).

## 12.3. RESULTS

Figures 12.1 to 12.6 show the lymphoproliferative responses of stored chicken and turkey blood to Con A. It was observed that the S.I. of 3 - 4-week old WLH chicken blood stored at RT for 48 hours and that stored at $4^0$C and RT for 72 hours were significantly lower than that of unstored blood (Figure 12.1). Blood collected from 5-week old WLH chicks (Figure 12.2), WLH layers in production (Figure 12.5), 3- and 5-week old broilers (Figures 12.3 and 12.4) and turkeys (Figure 12.6) could be kept only for 24 hrs at $4^0$C without significant reduction in S.I. compared to unstored blood.

Figures 12.7 and 12.8 show the mitogen responses to PWM of stored blood from 3 - 4-week old WLH chickens or turkey. It was observed that while chicken blood could only be stored for 24 hours at $4^0$C but not at RT without significant reduction in the mitogen responses (Figure 12.7), turkey blood could be stored either at $4^0$C or RT for 24 hrs (Figure 12.8).

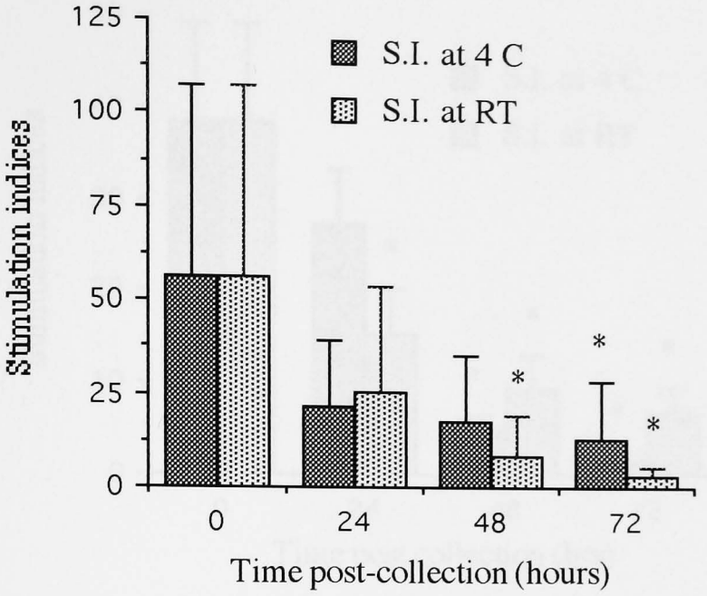


**Figure 12.1** Effect of storage of 3 - 4-week old WLH chicken blood on lymphocyte proliferative responses to Con A. (n = 8). Vertical bars indicate standard deviation. * indicates significant differences ($p < 0.05$, t - test ) in S.I. as compared to unstored blood.

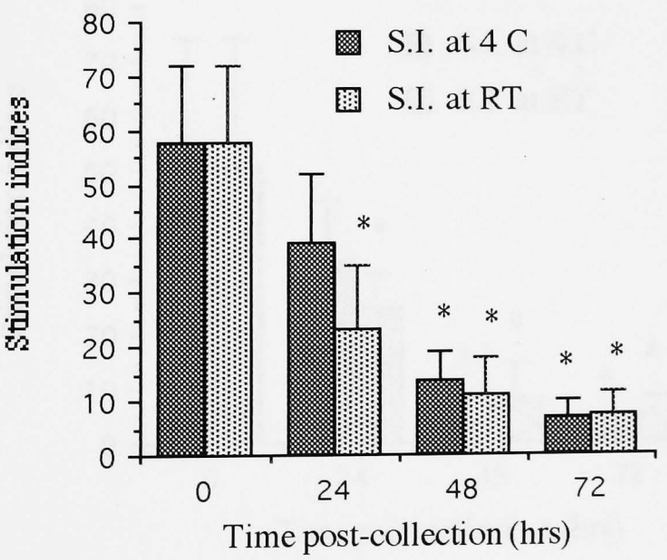


**Figure 12.2** Effect of storage of 5-week old WLH chicken blood on lymphocyte proliferative responses to Con A. (n = 5).

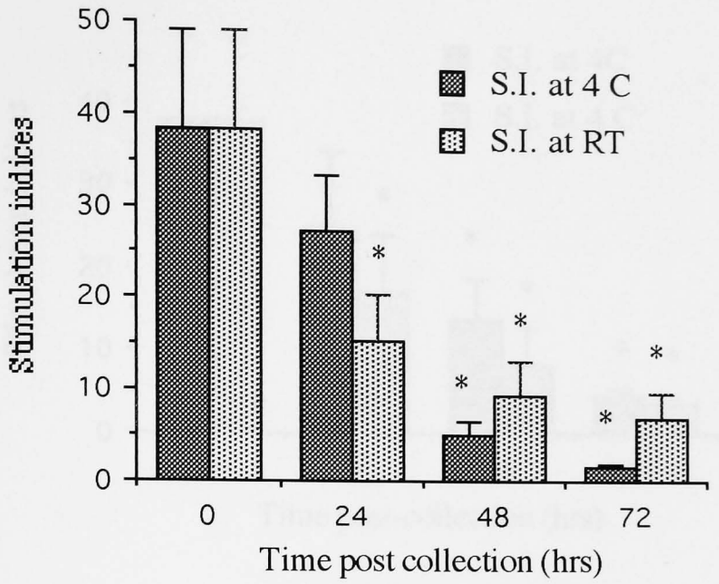


**Figure 12.3**. Effect of storage of 3-week old broiler-type chicken blood on lymphocyte proliferative responses to Con A. (n = 6).

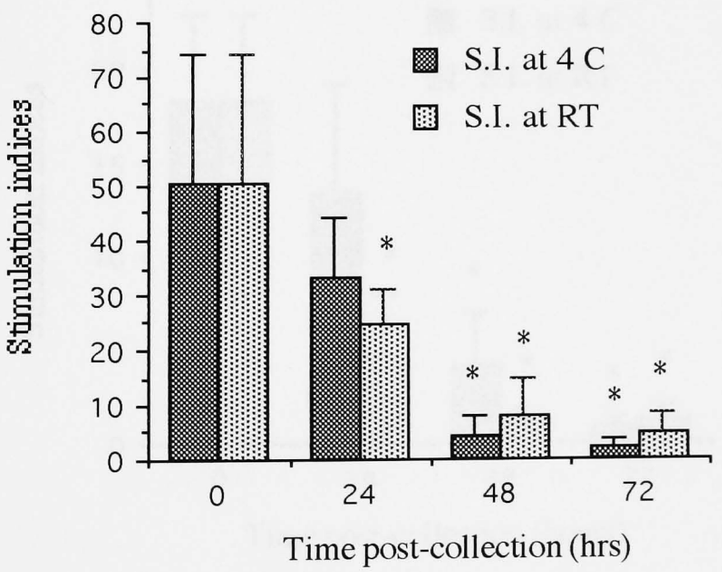


**Figure 12.4** Effect of storage of 5-week old broiler-type chicken blood on lymphocyte proliferative responses to Con A. (n = 6).

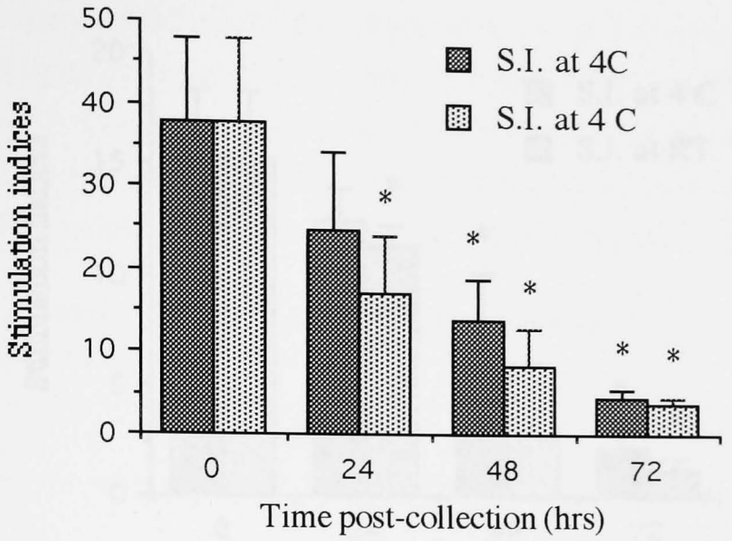


**Figure 12.5** Effect of storage of 48-week old WLH layer (in production) blood on lymphocyte proliferative responses to Con A. (n = 5).

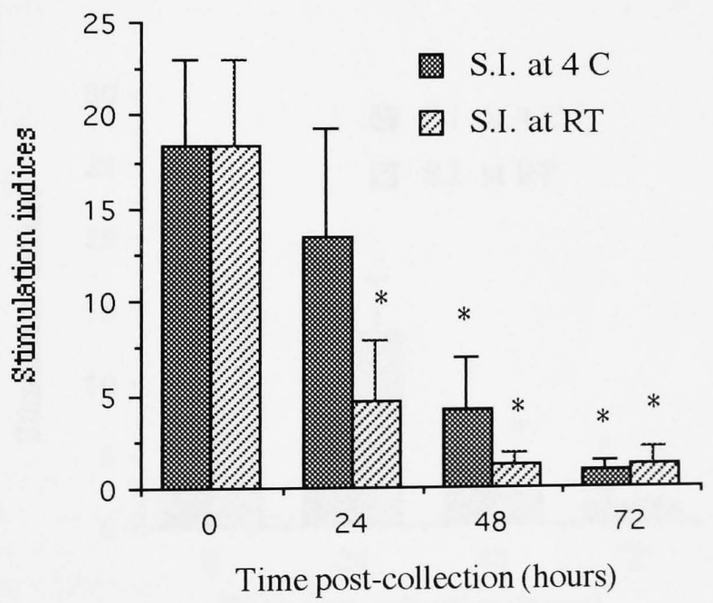


**Figure 12.6** Effect of storage of 3 - 4-week old turkey blood on lymphocyte proliferative responses to Con A. (n = 10).

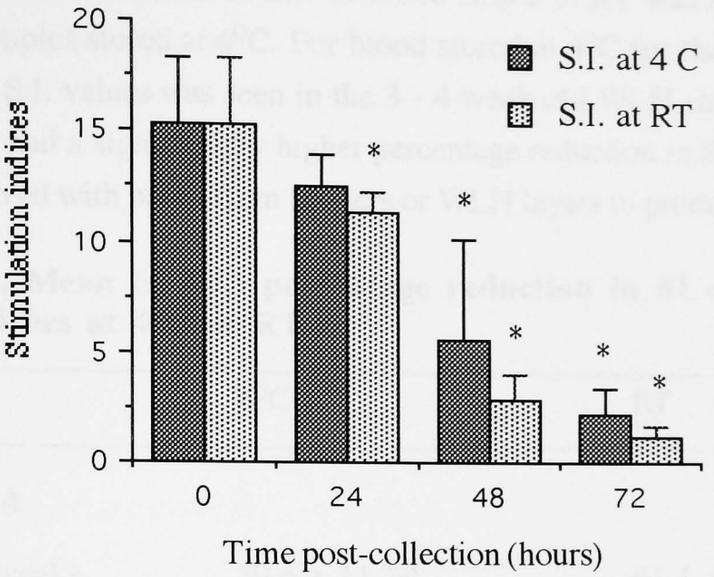


**Figure 12.7** Effect of storage of 3 - 4-week old WLH chicken blood on lymphocyte proliferative responses to PKM. (n = 8).

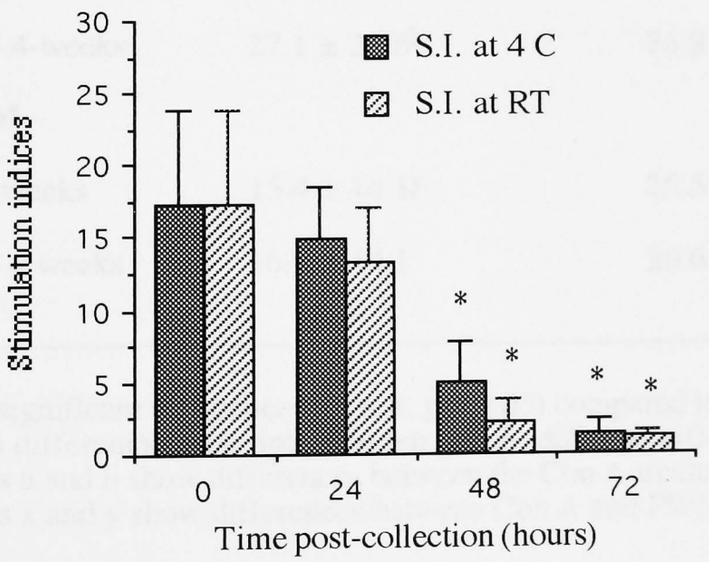


**Figure 12.8** Effect of storage of 3 - 4-week old turkey blood on lymphocyte proliferative responses to PKM. (n = 10).

The mean percentage reductions in S.I. values during the first 24 hrs storage of blood at $4^0$C or RT are presented in Table 12.1. For 5-week old WLH chickens, broilers and turkeys the mean reduction in S.I. of blood stored at RT was significantly greater than duplicate samples stored at $4^0$C. For blood stored at $4^0$C for the first 24 hrs, the highest reduction in S.I. values was seen in the 3 - 4-week old WLH chickens. The turkey blood stored at RT had a significantly higher percentage reduction in S.I. value compared to the values observed with blood from broilers or WLH layers in production.

**Table 12.1. Mean (± s.d.) percentage reduction in SI of blood stored for the first 24 hrs at $4^0$C or RT.**

| Birds | $4^0$C | RT |
|---|---|---|
| **With Con A** | | |
| WLH 3 - 4-weeks | $59.6 \pm 11.7^{ax}$ | $61.1 \pm 26.4^{x}$ |
| WLH 5-weeks | $33.0 \pm 10.2^{b}$ | $59.4 \pm 14.5^{*}$ |
| Broilers 3-weeks | $27.7 \pm 8.5^{b}$ | $59.8 \pm 4.4^{*a}$ |
| Broilers 5-weeks | $31.2 \pm 7.9^{b}$ | $51.1 \pm 13.4^{*a}$ |
| WLH Layers in production (48 weeks) | $35.4 \pm 11.4^{b}$ | $53.1 \pm 19.4^{a}$ |
| Turkeys 3 - 4-weeks | $27.1 \pm 20.6^{b}$ | $76.8 \pm 15.0^{*bx}$ |
| **With PWM** | | |
| WLH 3 - 4 weeks | $15.4 \pm 14.1^{y}$ | $25.5 \pm 14.0^{y}$ |
| Turkeys 3 - 4 weeks | $16.9 \pm 14.1$ | $20.6 \pm 11.4^{y}$ |

* indicates significant differences (t - test, $p < 0.05$) compared to blood stored at $4^0$C.
Values with different superscripts between groups differ significantly (t- test, $p< 0.05$).
Superscripts a and b show differences between the Con A treated groups.
Superscripts x and y show differences between Con A and PWM treated groups.

For 3 - 4-week old WLH chicken blood stored at $4^0$C, the mean reduction in S.I. to PWM was significantly lower than for Con A. For both 3 - 4-week old WLH chicken and turkey blood stored at RT, the mean reduction in S.I. values to PWM was significantly lower than those for Con A.

The S.I. values to Con A of unstored chicken blood were significantly higher than those of turkeys. The lymphoproliferative responses to Con A of blood obtained from 5-week old WLH chickens were significantly higher than those from 3-week old broilers and WLH layers in production. No significant differences were seen in S.I. to PWM of unstored blood obtained from 3 - 4-week old WLH chickens or turkeys.

## 12.4. DISCUSSION

The reduction in lymphoproliferative responses of stored blood may be attributed either to a reduction in numbers of responding lymphocytes or to alterations in their functional capacity to respond to mitogens. In general it was seen that, compared to unstored blood the S.I. of blood stored for 24 hrs at $4^0$C was not significantly reduced. However, the mean percentage reduction in S.I. values following stimulation with Con A during the first 24 hrs storage at $4^0$C varied from 27% for turkeys to 60% for 3 - 4-week old WLH chickens.

The reduction in proliferative responses to PWM of chicken blood during the first 24 hrs storage at $4^0$C was significantly lower compared to that seen with Con A. This may reflect differing alterations in the ratio of various cell types or earlier dysfunction of T-cells over B-cells. It is also known that accessory cells (monocytes) are required presentation of Con A while PKM can function without their presence (S. D. Carter, personal communication). Hence, death or dysfunction of monocytes during storage of blood could also contribute to the higher reduction in lymphoproliferative responses to Con A than PKM.

The S.I. values of unstored blood from 5-week old WLH chickens were significantly higher than those from 3-week old broilers, WLH layers in production and turkeys. The effect of age on lymphoproliferative assays using whole blood lymphocytes of chickens (Talebi et al., 1995) and turkeys (Sharma and Belzer, 1992) has been shown and it was seen that stimulatory response to Con A was dominant from three weeks onwards.

Thus for assessing mitogen responses of lymphocytes in whole blood cultures it is essential to keep the time between blood collection and culture to a minimum preferably with storage at $4^0$C. Over longer periods, considerations must be given to the reduction in S.I. during storage although the comparisons should be made with appropriate time-controlled samples.

# CHAPTER 13

## GENERAL DISCUSSION AND FUTURE WORK

This chapter is framed to discuss the salient features of the experimental results described in the previous chapters and to outline some possible future work. Various aspects of the pathogenicity and immunopathogenesis of IBV were examined in the experiments presented in this thesis.

Very little information is available in the literature regarding the comparative virulence of field and vaccine strains of IBV for the chicken oviduct. This may be due to the practical constraints of maintaining chickens till their oviducts become enlarged and differentiated to perform such studies. Peters et al. (1979) have shown that OOC prepared from different regions of the oviduct or differing ages of birds did not significantly alter the minimum infectious dose of the H52 vaccine virus for the tissue. Thus it appeared that if the amount of oviduct tissue obtained from young chicks could be increased it would be an easier and economical model system for studying interactions of IBV with the oviduct. Pradhan et al. (1984) used oestrogen treatment to increase the size of the oviducts obtained from young chicks. In this study, the OOC model system was further refined using culture conditions similar to that used for TOC. It was found that seven field strains of IBV and six commercial vaccine viruses multiplied in OOC as determined by various parameters such as virus titrations, IF and cessation of ciliary activity. Furthermore, it was shown that oestrogen treatment of chickens did not significantly alter virus titres of six commercial vaccines in TOC (Chapter 5) or the humoral and CMI responses (Chapter 7). Thus, it was confirmed that OOC prepared from precocious oviducts induced in young chicks by oestrogen treatment is an economical and reliable model for studying the effects of IBV on the oviducts.

The virulence of different isolates of mycoplasmas has been compared using scoring of ciliary activity and CAM concentrations as measures of virulence (Bermudez et al., 1990; Bermudez et al., 1992; Smits et al., 1994). The same criteria was applied for comparing the virulence of seven strains of IBV for TOC and OOC *in-vitro*. Minor differences between IBV strains in virulence for the oviducts were seen. Further studies are required using several other IBV strains belonging to different serotypes before adopting this model for *in-vitro* pathotyping of IBV viruses. Crinion and Hofstad (1972a) have suggested that two IBV strains, Connecticut and Iowa 609, were not pathogenic to chicken oviducts *in-vivo*. It would be interesting to test these two IBV strains in this model system. This model will also be useful in the study of

other viruses affecting the chicken oviduct such as NDV, Egg drop syndrome-76 virus or TRTV.

The assessment of ciliary activity in OOC, in terms of the percent of the periphery of rings having intact ciliary beating, was relatively difficult due to the presence of mucosal folds. So virus titres were used to measure the growth of six commercial vaccines in OOC and TOC in Chapter 5. Initially it was found that, for two of the most attenuated IBV vaccines available, the $CD_{50}$ titres in OOC were low compared to other vaccines tested. To justify this result, it was hypothesised that these two vaccine viruses multiply in OOC but all the multiplying virus populations do not produce ciliostasis. To confirm this, two other parameters for determining endpoints in virus titrations viz. IF and organ culture infectivity were examined. It was found that only for the two most attenuated vaccine viruses, titres determined by IF or organ culture infectivity were significantly higher than those determined by ciliostasis in both OOC and TOC. No such differences were seen in the titres determined by three different methods with virulent viruses. Hence it is proposed that differences in titres determined by ciliostasis or IF can be used as an *in-vitro* attenuation marker for IBV. Interestingly it was also seen that the differences in $CD_{50}$ and $IFID_{50}$ were higher for OOC than TOC which may indicate that IBV becomes attenuated earlier for oviducts than for trachea. Crinion and Hofstad (1972b) have shown that repeated embryo passage of IBV strain M41 reduced its virulence for chicken oviducts. It should be interesting to compare the same virulent virus at different passage levels using this method and to correlate the results with *in-vivo* virulence studies.

The ambitious attempt to prepare OOC from oestrogen-treated chicken embryos met with limited success as, neither was the size of the oviduct tissue greatly enlarged nor was uniform ciliary activity induced by these treatments. However, using IF as marker, it could be shown that IBV still multiplied in these tissues. Hence, this method of preparing OOC from chicken embryos could be used as a preliminary step to find out whether a particular virus multiplies in the oviduct tissue, as it obviates the need of maintaining chicks and treating them with oestrogen.

Local antibody production in the oviduct and gut of IBV-infected hens were shown for the first time in Chapter 6. The simple method of comparing antibody production at 37 $^0$C and 4 $^0$C as an indicator of active local antibody secretion is a valuable aid for studies involving mucosal immunity and would be an alternative to antibody-forming cell assays. Local antibody secretion was seen in the oviducts of IBV-infected hens in addition to antibodies transuded from the serum later in the course of the infection. This is the first ever evidence that, as in mammals, chicken oviducts are

also a component of the secretory immune system. Following IBV infection, the oviduct wash HI titres correlated well with egg production i.e. birds whose HI titres in oviduct washes were higher produced more eggs. If a simple method could be devised to obtain oviduct washes from live birds, it may be possible to predict whether that individual hen will lay normally or at a reduced level after a field exposure. Further work is required to determine the levels of coproantibodies induced by 'conventional' IBV strains and to determine their role in limiting the replication of IBV in the gut.

Conventional IBV vaccines for use in young chickens are aimed primarily at protecting the respiratory tract. No information is available on the protection afforded to the oviducts at that age. The effects of two vaccines and three virulent IBVs were studied in Chapter 7 primarily to ascertain protection at the oviduct level. An *in-vitro* challenge model, used by Hinze et al. (1991) was adopted since it was easy and also enabled the study of the importance of local antibodies in affording protection to the oviducts. Three virulent IBVs caused 100% ciliostasis in OOC at 3 weeks p.i., while the two vaccine viruses did not. This clearly indicates that examination of ciliary activity in OOC following vaccination is an alternative method to study attenuation of IBV vaccines for the oviduct. Presently, only histopathological examination of the oviducts is used as a criterion for this purpose.

At present, elaborate egg production trials are conducted to evaluate the efficacy of IBV vaccines in protecting the oviducts. These require long term experimentations and may suffer from lack of uniformity. Another potential new approach which this study has revealed is to use protection of ciliary activity in OOC following challenge, as a measure of efficacy of IBV vaccines in protecting the oviducts. A similar method is widely used to assess protection at the level of the trachea as it has been shown by several workers that there is complete agreement between ciliary activity, virus isolation and histopathological changes (Andrade et al. 1982; Marquardt et al. 1982; Snyder et al. 1983; Yachida et al., 1985). If vaccine-induced protection against structural damage (ciliostasis) is in agreement with protection against functional damage (drop in egg production), the former method can replace the existing latter method in safety and potency testing of IBV and also other virus vaccines which may affect oviducts. Further studies are required to validate this method.

The unexpected finding from this experiment was the heterologous protection offered by D274 vaccine to the new economically important variant IBV, 793/B which differs from it by 21% at the S1 amino acid sequence level (Adzhar et al., 1995). Although further *in-vivo* cross-protection studies are needed to confirm this finding, the message from this is that protectotypic studies are more useful than serotyping to

assess protection offered by existing vaccines to new serologically-distinct variants. It may also be possible that the fact that some new antigenic variants which arise in the field soon disappear (Cook, 1984), may be due to the cross-protection afforded by the existing vaccines, a factor which may have been inadvertantly overlooked. The use of the Archetti and Horsfall (1950) method to classify IBV strains into protectotypes based on ciliary scores following *in-vitro* challenge has enabled their clear-cut differentiation. It also facilitates direct comparisons between serotypes and protectotypes.

Thus it has been shown that OOC prepared from precocious oviducts induced by oestrogen treatment of young chicks are a valuable tool in the study of various aspects of virulence of IBV for the oviducts and immunity induced at the level of the oviduct by IBV vaccines.

The constant emergence of variant strains of IBV poses a serious threat to vaccination strategies. The new variant in the UK, variably designated as 793/B or 4/91 is still widely prevalent some 4 years after it was first reported and has warranted the production of a new vaccine against it. Field reports suggested several new manifestations of the disease caused by this variant strain of IBV. Hence an elaborate study was conducted on the immunopathogenesis of this virus in SPF chickens and six-week old broilers. The 793/B-like virus was found to be less pneumotropic and more enterotropic, even to the extent of causing diarrhoea. However, the spread and seroconversion appeared to be similar to conventional strains. Slight gross muscular changes were seen in experimentally infected broilers, which was never as severe as reported in the field, but a corresponding increase in serum CK levels were not recorded. Although the reason for such muscular changes was not elucidated in this study, a possible pathogenesis was suggested. The presence of IgM in the kidneys of infected chickens coincided with the days when the virus was not recovered from this organ. Hence it was postulated that this may be suggestive of immune complex formation and deposition in the kidneys. Although conjectural, it is possible that deposition of similar complexes in the capillary walls of muscles may lead to similar muscular changes seen in infected broilers. The formation of soluble immune complexes may also be related to the reported slow seroconversion, since the complexed antibodies may not be detected in antibody assays. Further studies are needed to unravel the pathogenesis of this extremely strange lesion seen with this variant IBV infection; the role of immune complexes may be one of the possible avenues which should be analysed in greater detail.

From here, the focus of the present study was shifted to immunity to IBV. The identification of lines of chickens resistant and sensitive to IBV has provided an excellent model for the study of immunity against IBV. Using this model, the role of humoral and local antibodies in resistance to IBV was studied by Cook et al. (1991a) and Cook et al. (1992). The role of cellular immunity was studied in Chapter 9 using CSP to suppress T-cells *in-vivo*. It was seen that a T-cell suppressed resistant line of chickens behaved like a sensitive line, in terms of mortality. Thus, it appears that T-cells protect chickens against IBV-induced mortality. The use of flow cytometry to determine T-cell subset changes following IBV infections in the resistant and sensitive line of chicken would throw more light on the class of T-cells involved in these effects. Another possible approach could be to selectively deplete specific T-cell subsets using Mab and examining its effect on the clinical disease.

Although IB is generally considered as an acute respiratory disease, prolonged virus excretion has been reported (Cook, 1968; Alexander et al., 1978; Chong and Apostolov, 1982). When day-old chicks were infected with an enterotropic IBV, strain G, faecal excretion of virus was not detected beyond day 35 p.i. but when birds reached sexual maturity, re-excretion occurred (Jones and Ambali, 1987). Virus re-excretion could not be induced earlier either by hormone injections (Ambali and Jones, 1991b) or by the application of social stress of rehousing with unfamiliar birds (Bhattacharjee et al., 1995). Virus re-excretion was successfully induced by treatment with CSP (Bhattacharjee et al., 1995) as assessed by virus recovery from tracheal and cloacal swabs. In this study, this experiment was repeated using the prototype virus M41 and virus isolation was done from various tissues after CSP treatment in an attempt to ascertain the site(s) of virus persistence. When two-week old chickens were infected with strain M41 and treated with CSP from 5 weeks p.i., no virus re-excretion was induced. In order to find out whether age at infection determines whether virus will persist, this experiment was repeated with chickens infected when one day of age. From the results of these trials, it was conclusively shown that kidneys are the site of virus persistence and that this is related to the age at infection.

The virus titres in the kidneys of CSP-treated chickens were very much higher than those in normal chickens. CSP-treatment of chickens previously infected with IBV could have caused similar increase in replication of the persistent virus enabling its isolation. Thus it seems that kidneys play a significant role in the immunopathogenesis of IBV infection, especially in the immunocompromised host. The cause of mortality in IBV-infected chickens is not exactly known but has been attributed to the physical blocking of the airways with plugs of mucus resulting in asphyxiation. Another possible cause which deserves further study is the contributory

factor of kidney damage to mortality even in non-nephropathogenic IBV infections. In countries where infectious bursal disease (IBD) is endemic, the IBV-induced kidney damage may be mistaken for IBD-induced lesions. Hence sufficient care must be exercised to differentiate the two.

In this study, T-cell suppression was induced artificially by injections of CSP. However, a similar sequelae could be expected with viruses like chicken anaemia virus or ingestion of immunosuppressive substances like mycotoxins which depress the T-cell system. Virus re-excretion from persistently infected chicks with no clinical signs would increase the probability of spread from infected to susceptible flocks in close proximity. Further studies are required to determine other factors which may induce re-excretion of persistently infected virus or infact vaccines and to unravel the nature of the re-excreted virus using molecular techniques.

To complete the study on the role of the different arms of the immune response in resistance to IBV, the role of non-specific immunity was investigated using a heteropaenic chicken model. Heterophils and their mammalian counterpart neutrophils are the least studied cells in virus infections. The development of a heteropaenic chicken model (Kogut et al., 1993) has enabled *in-vivo* studies to be performed to ascertain the importance of these cells in virus infections. It was observed that heterophils are ineffective in neutralising IBV but infact contribute to the damage of the tracheal epithelium. Nakamura et al. (1991) have shown that the damage to the tracheal epithelium was more severe and longer lasting in a sensitive line of chicken. It would be interesting to use this model to study the effect of heterophil depletion in the IBV-resistant line of chickens.

The role of macrophages in IBV infections also needs a more detailed study either using an *in-vivo* depletion model using carrageanean or by assessing macrophage function tests including the production of interleukin-1 and TNLF.

Although it is generally assumed that the S1 subunit of the spike protein is the likely inducer of protection in IBV-infected chickens, the role of other proteins in immunity to IBV remain unclear. Cross-reactive antibodies have been demonstrated against all the four structural proteins of IBV. All the proteins also elicit CMI responses but their cross-reactivity was studied in Chapter 11. It was shown that serotypically distinct strains of IBV induce cross-reactive responses that differed in magnitude with the strain of IBV. Further studies are required to identify epitopes which are involved in the generation of cross-reactive humoral or CMI responses. This might enable the

production of a broad-spectrum vaccine effective against all IBV strains. This is the area in which the future work on IBV should be directed.

At present, no easy and convenient assays are available to assess cellular immunity in chickens. Hence, all the poultry vaccines are tested with particular emphasis on the humoral antibody responses even though with some diseases it has been clearly shown that the levels of antibodies do not correlate with protection. In the case of IBV, protection is assessed based on scoring of tracheal ciliary activity or fate of the intact bird, following challenge with IBV and/or *E.coli.* Both these challenge models measure local protection at the level of the trachea.

Recently it has been shown that virulent IBV caused transient immunosuppression, as ascertained by lymphoproliferation responses of whole blood to PHA (Wakenell et al., 1995) and it was also shown that IBV vaccine protected the bird against this challenge virus-induced immunosuppression. Similar transient reduction in mitogen responses to another T-cell mitogen Con A was seen in chickens infected with IBV strain M41 (Chapter 9). It may be useful to include this parameter in future while examining the efficacy of IBV vaccines.

In order to apply lymphoproliferation assays to flock situations, it is essential to know the effect of storage of blood on lymphoproliferative responses to plant lectins. This area was examined in Chapter 12. It was found that chicken and turkey blood could be stored at $4^0$C only for 24 hours without significant reduction in lymphoproliferative responses to Con A or PWM. This information would be of some use while applying this assay under field conditions.

Thus, several areas of pathogenicity and immunopathogenesis of IBV have been examined. The work has thrown open more questions than providing answers and it requires much more work to unravel the mystery of this 80 nm particle. *The viruses may always remain one step ahead of human endeavour.*

# REFERENCES

ADAIR, B. M., MCNEILLY, F., MCCONNELL, C. D. G., TODD, D., NELSON, R. T. AND MCNULTY, M. S. (1991) Effects of chicken anaemia agent on lymphokine production and lymphocyte transformation in experimentally infected chickens. *Avian Diseases* **35** : 783 - 792

ADLER, H. E., MCMARTIN, D. A. AND ORTAMAYER, H. (1962) The effect of infectious bronchitis virus on chickens infected with *Mycoplasma gallisepticum* . *Avian Diseases* **6** : 267 - 274

ADZHAR, A. B., SHAW, K., BRITTON, P AND CAVANAGH, D. (1995) Avian infectious bronchitis virus : differences between 793/B and other strains. *Veterinary Record* **136** : 548

AFANADOR, G. AND ROBERTS, J. R. (1994) Effect of nephropathogenic infectious bronchitis virus on renal function in male broiler chickens. *British Poultry Science* **35** : 445 - 456

ALBASSAM, M. A., WINTERFIELD, R. W. AND THACKER, H. L. (1986) Comparison of the nephropathogenicity of four strains of infectious bronchitis virus. *Avian Diseases* **30** : 468 - 476

ALBINI, B., WICK, G., ROSE, M. E. AND ORLANS, E. (1974) Immunoglobulin production in chicken Harderian glands. *International Archives of Allergy and Applied Immunology* **47** : 23 - 34

ALEXANDER, D. J. AND GOUGH, R. E. (1977) Isolation of avian infectious bronchitis virus from experimentally infected chickens. *Research in Veterinary Science* **23** : 344 - 347

ALEXANDER, D. J., GOUGH, R. E. AND PATTISON, M. (1978) A long-term study of the pathogenesis of infection of fowls with three strains of avian infectious bronchitis virus. *Research in Veterinary Science* **24** : 227 - 233

AMBALI, A. G. AND JONES, R. C. (1990) Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. *Avian Diseases* **34** : 809 - 817

AMBALI, A. G. AND JONES, R. C. (1991a) Effects of trypsin and sodium tauroglycocholate on an enterotropic variant infectious bronchitis virus. *Veterinary Record* **129** : 510 - 511

AMBALI, A. G. AND JONES, R. C. (1991b) The effects of oestrogen and progesterone on re-excretion of infectious bronchitis virus strain G in specific-pathogen-free chickens. *Journal of Hygiene, Epidemiology, Microbiology and Immunology* **35** : 429 - 439

ANDRADE, L. F., VILLEGAS, P., FLETCHER, O. J. AND LAUNDENCIA, R. (1982) Evaluation of ciliary movement in tracheal rings to assess immunity against infectious bronchitis virus. *Avian Diseases* **26** : 805 - 815

ANDRADE, L. F., VILLEGAS, P. AND FLETCHER, O. J. (1983) Vaccination of day-old broilers against infectious bronchitis: effect of vaccine strain and route of administration. *Avian Diseases* **27** : 178 - 187

ANDREASEN, J. R., JACKWOOD, M. W. AND HILT, D. A. (1991) Polymerase chain reaction amplification of the genome of infectious bronchitis virus. *Avian Diseases* **35** : 216 - 220

ANDREASEN, C. B., LATIMER, K. S. AND STEFFANS, W. L. (1990) Evaluation of chicken heterophil adherence. *Avian Diseases* **34** : 639 - 642

ANIMAS, S. B., OTSUKI, K., TSUBOKURA, M. AND COOK, J. K. A. (1994a) Comparison of the susceptibility of chicks of different ages to infection with nephrosis-nephritis causing strain of infectious bronchitis virus. *Journal of Veterinary Medical Science* **56** : 449 - 453

ANIMAS, S. B., OTSUKI, K., HANAYAMA, M., SANEKATA, T. AND TSUBOKURA, M. (1994b) Experimental infection with avian infectious bronchitis virus (Kagoshima-34 strain) in chicks of different ages. *Journal of Veterinary Medical Science* **56** : 443 - 447

ARCHETTI, I. AND HORSFALL, F. L. (1950) Persistent antigenic variation of influenza viruses after incomplete neutralisation *in-ovo* with heterologous immune serum. *Journal of Experimental Medicine* **92** : 411 - 462

ARNOLD, J. W. AND HOLT, P. S. (1995) Response to *Salmonella enteritidis* infection by the immunocompromised avian host. *Poultry Science* **74** : 656 - 665

ARVIDSON, Y., TANNOCK, G. A., SENTHILSELVAN, A. AND ZERBES, M. (1990) A model for determining immunogenic relationships between avian infectious bronchitis virus. *Archives of Virology* **111** : 227 - 238

ASAI, T., OKADA, M., ONO, M., IRISAWA, T, MORI, Y., YOKOMIZO,Y. AND SATO, S. (1993) Increased levels of tumour necrosis factor and interleukin-1 in bronchoalveolar lavage fluids from pigs infected with *Mycoplasma hyopneumoniae*. *Veterinary Immunology and Immunopathology* **38** : 253 - 260

AVELLANDA, G. A., VILLEGAS, P., JACKWOOD, M. W. AND KING, D. J. (1994) *In-vivo* evaluation of the pathogenicity of field isolates of infectious bronchitis virus. *Avian Diseases* **38** : 589 - 597

BABA, T., MASUMOTO, K., NISHIDA, S. , KAJIKAWA, T. AND MITSUI, M. (1988) Harderian gland dependency of immunoglobulin A production in the lachrymal fluid of chickens. *Immunlogy* **65** : 67 - 71

BABA, T., KAWATA, T., MASUMOTO, K. AND KAJIKAWA, T. (1990) Role of Harderian gland in immunoglobulin A production in chicken lachrymal fluid. *Research in Veterinary Science* **49** : 20 - 24

BARR, D. A., REECE, R. L., O' ROURKE, D., BUTTON, C. AND FARAGHER, J. T. (1988) Isolation of infectious bronchitis virus from a flock of racing pigeons. *Australian Veterinary Journal* **65** : 228

BEAUDETTE, F. R. AND HUDSON, C. B. (1937) Cultivation of the virus of infectious bronchitis. *Journal of the American Veterinary Medical Association* **90** : 51 - 60

BERMUDEZ, V. M., MILLER, R. B., ROSENDAL, S., FERNANDO, M. A., JOHNSON, W. H. AND O'BRIEN, P. J. (1992) Measurement of the cytotoxic effects of different strains of *Mycoplasma equigenitalium* on the equine uterine tube using a calmodulin assay. *Canadian Journal of Veterinary Research* **56** : 331 - 338

BERMUDEZ, V. M., MILLER, R. B., ROSENDAL, S. AND JOHNSON, W. (1990) *In-vitro* cytopathic effect of *Mycoplasma equigenitalium* on the equine uterine tube. In: Recent advances in Mycoplasmology. Eds. Stanek, G., Cassel, G. H., Tully, J. H., Whitcomb, R. F. *Proceedings of the Seventh Congress of the International Organisation of Mycoplasmology*, Baden, Near Vienna, 1988; Supplement **20** : 419 - 428

BERRY, D. M., CRUICKSHANK, J. G., CHU, H. P. AND WELLS, R. J. H. (1964) The structure of infectious bronchitis virus. *Virology* **23** : 403 - 407

BHATTACHARJEE, P. S. (1994) Enterotropism and persistence of avian infectious bronchitis virus in chickens. *Ph.D. Thesis*. University of Liverpool.

BHATTACHARJEE, P. S., CARTER, S. D., SAVAGE, C. E. AND JONES, R. C. (1995) Re-excretion of infectious bronchitis virus in chickens induced by cyclosporin. *Avian Pathology* **24** : 435 - 441

BHATTACHARJEE, P. S., NAYLOR, C. J. AND JONES, R. C. (1994) A simple method for immunofluorescence staining of tracheal organ cultures for the rapid identification of infectious bronchitis virus. *Avian Pathology* **23** : 471 - 480

BINNS, M. M. (1988) Infectious bronchitis virus: its genome and protein structure. *Proceedings of the Ist International Symposium on Infectious bronchitis*, Rauischholzhausen, Germany 141 - 144

BINNS, M. M., BOURSNELL, M. E. G., TOMLEY, F. M. AND BROWN, T. D. K. (1986) Nucleotide sequence encoding the membrane protein of the IBV strain 6/82. *Nucleic Acids Research* **14** : 5558.

BOMBARA, C. J. AND TAYLOR, R. L. JR. (1991) Signal transduction events in chicken interleukin-1 production. *Poultry Science* **70** : 1372 - 1380

BOOTS, A. M. H. (1991) The cellular immune response to infectious bronchitis virus: A model for the development of a peptide based vaccine. *Ph.D thesis*. University of Utrecht.

BOOTS, A. M. H., KUSTERS, J. G., VAN NOORT, J. M., ZWAAGSTRA, K. A., RIJKE, E., VAN DER ZEIJST, B. A. M. AND HENSEN, E. J. (1991) Localisation of a T-cell epitope within the nucleocapsid protein of the avian corona virus. *Immunology* **74** : 8 - 13

BOOTS, A. M. H., BENAISSA-TROUW, B. J., HESSELINK, W., RIJKE, E., SCHRIER, C. AND HENSEN, E. J. (1992) Induction of antiviral immune responses by immunisation with recombinant-DNA encoded avian corona virus nucleocapsid protein. *Vaccine* **10** : 119 - 124

BOX, P. G., BERESFORD, A. V. AND ROBERTS, B. (1980) Protection of laying hens against infectious bronchitis with inactivated emulsion vaccines. *Veterinary Record* **106** : 264 - 268

BOX, P. G. AND ELLIS, K. R. (1985) Infectious bronchitis in laying hens: interference with response to emulsion vaccine by attenuated live vaccine. *Avian Pathology* **14** : 9 - 22

BOX, P. G., HOLMES, H. C., FINNEY, P. M. AND FROYMANN, R. (1988) Infectious bronchitis in laying hens: the relationship between haemagglutination-inhibition antibody levels and resistance to experimental challenge. *Avian Pathology* **17** : 349 - 361

BREIDER, M. A., WALKER, R., HOPKINS, F., SCHULTZ, T. AND BOWERSOCK, T. (1988) Pulmonary lesions induced by *Pasteurella haemolytica* in neutrophil sufficient and neutrophil deficient calves. *Canadian Journal of Veterinary Research* **52** : 205 - 209

BRETON-GORIUS, J., COQUIN, Y. AND GUICHARD, J. (1978) Cytochemical distinction between azurophils and catalase-containing granules in leukocytes. I. Studies in developing neutrophils and monocytes from patients with myeloperoxidase deficiency: comparison with peroxidase deficient chicken heterophils. *Laboratory Investigation* **38** : 21 - 31

BROWN, A. J. AND BRACEWELL, C. D. (1985) Application of haemagglutination-inhibition test to typing of infectious bronchitis viris. *Veterinary Record* **116** : 47 - 48

BROWN, A. J. AND BRACEWELL, C. D. (1988) Effect of repeated infections of chickens with infectious bronchitis viruses on the specificity of their antibody responses. *Veterinary Record* **122**: 207 - 208

BRUNE, K. AND SPITZNAGEL, J. (1973) Peroxidase-less chicken leukocytes : isolation and characterisation of antibacterial granules.*Journal of Infectious Diseases* **127** : 84 - 94

BUMSTEAD, N., HUGGINS, M. B. AND COOK, J. K. A. (1989) Genetic differences in susceptibility to a mixture of avian infectious bronchitis virus and *Escherichia coli*. *British Poultry Science* **30** : 39 - 48

BUTCHER, G. D., WINTERFIELD, R. W. AND SHAPIRO, D. P. (1990) Pathogenesis of H13 nephropathogenic infectious bronchitis virus. *Avian Diseases* **34** : 916 - 921

BUTLER, E. J., CURTIS, M. J., PEARSON, A. W. AND MCDOUGALL, J. S. (1972a) Effect of infectious bronchitis virus on the structure and composition of egg albumen. *Journal of the Science of Food and Agriculture* **23** : 359 - 369

BUTLER, M., ELLAWAY, W. J. AND HALL, T. (1972b) Comparative studies on the infectivity of avian respiratory viruses for eggs, cell culture and tracheal explants. *Journal of Comparative Pathology* **82** : 327 - 332

BYRNES, S., EATON, R. AND KOGUT, M. (1993) *In-vitro* interleukin-1 and tumour necrosis factor production by macrophages from chickens infected with *Eimeria maxima* or *Eimeria tenella*. *International Journal of Parasitology* **23** : 639 - 645

CADMAN, H. F., KELLY, P. J., ZHOU, R., DAVELAAR, F. AND MASON, P. R. (1994) A serosurvey using enzyme linked immunosorbent assay for antibodies against poultry pathogens in Ostriches (Struthio-camelus) from Zimbabwe. *Avian Diseases* **38** : 621 - 625

CAMPBELL, T. W. (1988) Avian haematology and cytology. Iowa State University Press, Ames, Iowa.

CAPUA, I., GOUGH, R. E., MANCINI, M., CASACCIA, C. AND WEISS, C. (1994) A novel infectious bronchitis strain infecting broiler chickens in Italy. *Journal of Veterinary Medicine* Series B **41** : 83 - 89

CASSATELLA, M. A. (1995) The production of cytokines by polymorphonuclear neutrophils. *Immunology Today* **16** : 21 - 26

CAVANAGH, D. (1983a) Coronavirus IBV glycopolypeptides: size of their polypeptide moieties and nature of their oligosaccharides. *Journal of General Virology* **64** : 1187 - 1191

CAVANAGH, D. (1983b) Coronavirus IBV: further evidence that the surface projections are associated with two glycopolypeptides. *Journal of General Virology* **64** : 1787 - 1791

CAVANAGH, D. (1983c) Coronavirus IBV: structural characterisation of the spike protein. *Journal of General Virology* **64** : 2577 - 2583

CAVANAGH, D., ADZHAR, A. B., BRITTON, P., KOTTIER, S., MAWDITT, K., NAYLOR, C., PENZES, Z. AND SHAW, K. (1995) Molecular approaches to the diagnosis and prophylaxis of avian diseases. *Asia-pacific Journal of Molecular Biology and Biotechnology* **3** : 272 - 279

CAVANAGH, D., DARBYSHIRE, J. H., DAVIS, P. AND PETERS, R. W. (1984) Induction of humoral neutralising and haemagglutination-inhibiting antibody by the spike protein of infectious bronchitis virus. *Avian Pathology* **13** : 573 - 583

CAVANAGH, D. AND DAVIS, P. J. (1987) Coronavirus IBV: relationships among recent European isolates studied by limited proteolysis of the virion glycopolypeptides. *Avian Pathology* **16** : 1 - 13

CAVANAGH, D. AND DAVIS, P. J. (1988) Evolution of avian coronavirus IBV: sequence of the matrix glycoprotein gene and intergenic region of several serotypes. *Journal of General Virology* **69** : 621 - 629

CAVANAGH, D., DAVIS, P. J., COOK, J. K. A., LI, D., KANT, A. AND KOCH, G. (1992a) Location of amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. *Avian Pathology* **21** : 33 - 43

CAVANAGH, D., DAVIS, P. J. AND COOK, J. K. A. (1992b) Infectious bronchitis virus: evidence for recombination within the Massachusetts serotype. *Avian Pathology* **21** : 401 - 408

CAVANAGH, D., DAVIS, P. J., DARBYSHIRE, J. H. AND PETERS, R. W. (1986) Coronavirus IBV : virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralising of hamagglutination-inhibiting antibody or induce chicken tracheal protection. *Journal of General Virology* **67** : 1435 - 1442

CHAN, M. M., CHEN, C. L. H., AGER, L. L. AND COOPER, M. D. (1988) Identification of the avian homologue of mammalian CD4 and CD8 antigens. *Journal of Immunology* **140**: 2133 - 2138

CHANDRA, M. (1987) Comparative nephropathogenicity of different strains of infectious bronchitis virus in chickens. *Poultry Science* **66** : 954 - 959

CHANDRA, M. (1988) Comparative nephropathogenicity of infectious bronchitis virus in bursectomised and non-bursectomised chickens. *American Journal of Veterinary Research* **49** : 831 - 834

CHANG, C. F. AND HAMILTON, P. B. (1979) Impaired phagocytosis by heterophils from chicks during aflatoxicosis. *Toxicology and Applied Pharmacology* **48** : 459 - 466

CHEN, B. Y., HOSI, S., NUNOYA, T. AND ITAKURA, C. (1996) Histopathology and immunochemistry of renal lesions due to infectious bronchitis virus in chickens. *Avian Pathology* **25** : 269 - 283

CHEUNG, W. Y. (1982) Calmodulin. *Scientific American* **246** : 62 - 70

CHONG, K. T. AND APOSTOLOV, K. (1982) The pathogenesis of nephritis in chickens induced by infectious bronchitis virus. *Journal of Comparative Pathology* **92** : 199 - 211

CHUBB, R. C. (1973) The effectiveness of vaccination against the Australian infectious bronchitis nephritis virus before two weeks of age. *Veterinary Record* **93** : 249 - 252

CHUBB, R. C. (1974) The effect of suppression of circulating antibody on resistance to the Australian avian infectious bronchitis virus. *Research in Veterinary Science* **17** : 169 - 173

CHUBB, R. C., HUYNH, V. AND LAW, R. (1987) The detection of cytotoxic lymphocyte activity in chickens infected with infectious bronchitis virus or fowlpox virus. *Avian Pathology* **16** : 395 - 405

CHUBB, R. C., HUYNH, V. AND BRADLEY, R. (1988) The induction and control of delayed type hypersensitivity reactions induced in chickens by infectious bronchitis virus. *Avian Pathology* **17** : 371 - 383

CLARKE, J. K., MCFERRAN, J. B. AND GAY, F. W. (1972) Use of allantoic cells for the detection of avian infectious bronchitis virus. *Archiv fur die Gesamte Virusforschung* **36** : 62 - 70

CLOUD, S. S., ROSENBERGER, J. K. AND LILLEHOJ, H. S. (1992) Immune dysfunction following infection with chicken anaemia agent and infectious bursal disease virus. II. Alterations of *in-vitro* lymphoproliferation and *in-vivo* immune responses. *Veterinary Immunology and Immunopathology* **34** : 353 - 366

CLEWLEY, J. P., MORSER, J., AVERY, R. J. AND LOMNICZI, B. (1981) Oligonucleotide fingerprinting of ribonucleic acids of infectious bronchitis virus strains. *Infection and Immunity* **32** : 1227 - 1233

CONDRON, R. J. AND MARSHALL, A. T. (1985) Pathogenesis of infectious bronchitis nephritis. 2. Studies of water and electrolyte balance in colostomised chickens. *Avian Pathology* **14** : 509 - 520

CONDRON, R. J. AND MARSHALL, A. T. (1986) Pathogenesis of infectious bronchitis nephritis 1. Morphometric analysis of kidney proximal tubular epithelium in chickens. *Journal of Comparative Pathology* **96** : 47 - 61

COOK, J. K. A. (1968) Duration of experimental infectious bronchitis in chickens. *Research in Veterinary Science.* **9** : 506 - 514

COOK, J. K. A. (1971) Recovery of infectious bronchitis virus from eggs and chicks produced by experimentally inoculated hens. *Journal of Comparative Pathology* **81** : 203 - 211

COOK, J. K. A. (1983) Isolation of a new serotype of infectious bronchitis-like virus from chickens in England. *Veterinary Record* **112** : 104 - 105

COOK, J. K. A. (1984) The classification of new serotypes of infectious bronchitis virus isolated from poultry flocks in Britain between 1981 and 1983. *Avian Pathology* **13** : 733 - 741

COOK, J. K. A., BROWN, A. J. AND BRACEWELL, C. D. (1987) Comparison of the haemagglutination-inhibition test and the serum neutralisation test in tracheal organ cultures for typing infectious bronchitis virus strains. *Avian Pathology* **16** : 505 - 511

COOK, J. K. A., DARBYSHIRE, J. H. AND PETERS, R. W. (1976) The use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. *Archives of Virology* **50** : 109 - 118

COOK, J. K. A., DAVISON, T. F., HUGGINS, M. B. AND MCLAUGHLAN, P. (1991a) Effect of *in-ovo* bursectomy on the course of an infectious bronchitis virus infection in line C white leghorn chickens. *Archives of Virology* **118** : 225 - 234

COOK, J. K. A. AND ELLIS, M. M. (1990) Attenuation of turkey rhinotracheitis virus by alternative passage in embryonated chicken eggs and tracheal organ culture. *Avian Pathology* **19** : 181 - 185

COOK, J. K. A., ELLIS, M. M., DOLBY, C. A., HOLMES, H. C., FINNEY, P. M. AND HUGGINS, M. B. (1989) A live attenuated turkey rhinotracheitis virus vaccine. 1. Stability of the attenuated strain. *Avian Pathology* **18 :** 511 - 522

COOK, J. K. A. AND HUGGINS, M. B. (1986) Newly isolated serotypes of infectious bronchitis virus: their role in disease. *Avian Pathology* **15** : 129 - 138

COOK, J. K. A., HUGGINS, M. B. AND ELLIS, M. M. (1991b) Use of an infectious bronchitis virus and *Escherichia coli* model infection to assess the ability to vaccinate sucessfully against infectious bronchitis in the presence of maternally-derived immunity. *Avian Pathology* **20** : 619 - 626

COOK, J. K. A., OTSUKI, K., DA SILVA MARTINS, N. R., ELLIS, M. M. AND HUGGINS, M. B. (1992) The secretory antibody response in inbred lines of chickens to avian infectious bronchitis virus infection. *Avian Pathology* **21** : 681 - 692

COOK, J. K. A., ORBELL, S. J., WOODS, M. A. AND HUGGINS, M. B. (1996) A survey of the presence of a new infectious bronchitis virus designated 4/91 (793B). *Veterinary Record* **138** : 178 - 180

COOK, J. K. A., SMITH, H. W. AND HUGGINS, M. B. (1986) Infectious bronchitis immunity: its study in chickens experimentally infected with mixtures of infectious bronchitis virus and *Escherichia coli*. *Journal of General Virology* **67** : 1427 - 1434

CRINION R. A. P., BALL, R. A. AND HOFSTAD, M. S. (1971a) Pathogenesis of oviduct lesions in immature chickens following exposure to infectious bronchitis virus at one day-old. *Avian Diseases* **15** : 32 - 41

CRINION R. A. P., BALL, R. A. AND HOFSTAD, M. S. (1971b) Abnormalities in laying chickens following exposure to infectious bronchitis virus at one day-old. *Avian Diseases* **15** : 42 - 48

CRINION, R. A. P. AND HOFSTAD, M. S. (1972a) Pathogenicity of four serotypes of avian infectious bronchitis virus for the oviduct of young chickens of various ages. *Avian Diseases* **16** : 351 - 363

CRINION, R. A. P. AND HOFSTAD, M. S. (1972b) Pathogenicity of two embryo-passage levels of avian infectious bronchitis virus for the oviduct of young chickens of various ages. *Avian Diseases* **16** : 967 - 973

CSERMELYI, M., THIJSSEN, R., ORTHEL, F., BURGER, A. G., KOUWENHOVEN, B. AND LUTTICKEN, D. (1988) Serological classification of recent infectious bronchitis virus isolates by the neutralisation of immunofluorescent foci. *Avian Pathology* **17** : 139 - 148

CUBILLOS, A., ULLOA, J., CUBILLOS, V. AND COOK, J. K. A. (1991) Characterisation of strains of infectious bronchitis virus isolated in Chile. *Avian Pathology* **20** : 85 - 99

CUMMING, R. B. (1962) The etiology of uraemia of chickens. *Australian Veterinary Journal* **38** : 554

CUMMING, R. B. (1963) Infectious avian nephrosis (uraemia) in Australia. *Australian Veterinary Journal* **39** : 145 - 147

CUMMING, R. B. (1969) The control of avian infectious bronchitis/nephrosis in Australia. *Australian Veterinary Journal* **45** : 200 - 203

CUMMING, R. B. AND CHUBB, R. C. (1988) The pathogenesis of nephritis evoked by Australian IB viruses. *Proceedings of the Ist International Symposium on Infectious Bronchitis,* Rauischholzhausen, Germany, pp. 129 - 132

CUNNINGHAM, C. H. (1970) Avian infectious bronchitis. *Advances in Veterinary Science and Comparative Medicine* **14**: 105 - 148

CUTURI, M. C., MURPHY, M., COSTA-GIOMI, M. P., WEINMANN, R., PERUSSIA, B. AND TRINCHIERI, G. (1987) Independent regulation of tumour necrosis factor and lymphocytes. *Journal of Experimental Medicine* **165**: 1581 - 1594

DARBYSHIRE, J. H. (1980) Assessment of cross-immunity in chickens to strains of avian infectious bronchitis virus using tracheal organ cultures. *Avian Pathology* **9** : 179 - 184

DARBYSHIRE, J. H. (1985) A clearance test to assess protection in chickens vaccinated against infectious bronchitis virus. *Avian Pathology* **14** : 497 - 508

DARBYSHIRE, J. H., COOK, J. K. A. AND PETERS, R. W. (1976) Organ culture studies on the efficiency of infection of chicken tissues with avian infectious bronchitis virus. *British Journal of Experimental Pathology* **57** : 443 - 454

DARBYSHIRE, J. H., COOK, J. K. A. AND PETERS, R. W. (1978) Growth comparisons of avian infectious bronchitis virus strains in organ cultures of chicken tissues. *Archives of Virology* **56**: 317 - 325

DARBYSHIRE, J. H. AND PETERS, R. W. (1984) Sequential development of humoral immunity and assessment of protection in chickens following vaccination and challenge with avian infectious bronchitis virus. *Research in Veterinary Science* **37** : 77 - 86

DARBYSHIRE, J. H. AND PETERS, R. W. (1985) Humoral antibody response and assessment of protection following primary vaccination of chicks with maternally-derived antibody against avian infectious bronchitis virus. *Research in Veterinary Science* **38**: 14 - 21

DARBYSHIRE, J. H., ROWELL, R. G., COOK, J. K. A. AND PETERS, R. W. (1979) Taxonomic studies on strains of avian infectious bronchitis virus using neutralisation tests in tracheal organ cultures. *Archives of Virology* **61**: 227 - 238

DAVELAAR, F. G. AND KOUWENHOVEN, B. (1976) Changes in the Harderian gland of the chicken following conjunctival and intranasal infection with infectious bronchitis virus in one and 20 day-old chickens. *Avian Pathology* **5** : 39 - 50

DAVELAAR, F. G. AND KOUWENHOVEN, B. (1977) Influence of maternal antibodies on vaccination of chicks of different ages against infectious bronchitis virus. *Avian Pathology* **6** : 41 - 50

DAVELAAR, F. G. AND KOUWENHOVEN, B. (1980) Effect of removal of the Harderian gland in one day-old chicks on immunity following infectious bronchitis vaccination. *Avian Pathology* **9** : 489 - 497

DAVELAAR, F. G. AND KOUWENHOVEN, B. (1981) Study on the local effect of eye-drop vaccination against infectious bronchitis in one day-old chicks with maternal antibodies. *Avian Pathology* **10** : 83 - 90

DAVELAAR, F. G., KOUWENHOVEN, B. AND BURGER, A. G. (1984) Occurence and significance of infectious bronchitis virus variant strains in egg and broiler production in the Netherlands. *Veterinary Quarterly* **6** : 114 - 120

DAVELAAR, F. G., NOORDZIJ, A. AND VAN DER DONK, J. A. (1982) A study on the synthesis and secretion of immunoglobulins by the Harderian gland of fowl after eye-drop vaccination against infectious bronchitis at 1 day-old. *Avian Pathology* **11** : 63 - 79

DAVELAAR, F. G. AND VANDENBOS, J. (1992) Ascorbic acid and infectious bronchitis infections in broilers. *Avian Pathology* **21** : 581 - 589

DE WIT, J. J., DAVELAAR, F. G. AND BRAUNIUS, W. W. (1992) Comparison of the enzyme linked immunosorbent assay, the haemagglutination-inhibition test and agar gel precipitation test for the detection of antibody against infectious bronchitis and Newcastle disease in commecial broilers. *Avian Pathology* **21** : 651 - 658

DEWIT, J. J., KOCH, G., KANT, A. AND VAN ROOZELAAR, D. J. (1995) Detection by immunofluorescent assay of serotype-specific and group-specific antigens of infectious bronchitis virus in tracheas of broilers with respiratory disease. *Avian Pathology* **24** : 465 - 474

DROUAL, R. AND WOOLCOCK, P. R. (1994) Swollenhead syndrome associated with *Escherichia coli* and infectious bronchitis virus in the Central Valley of California. *Avian Pathology* **23** : 733 - 742

EL HOUADFI, M. D., EL BOUZIDI, H., NAJAT OUALIT, JONES, R. C., JORDAN, F. T. W. AND COOK, J. K. A. (1991) Enterotropic infectious bronchitis (strain G) in layers. In: *Proceedings of the IInd International Symposium on Infectious Bronchitis.* Rauischholzhausen, Germany. pp. 127 - 136

EL HOUADFI, M. D., JONES, R. C., COOK, J. K. A. AND AMBALI, A. G. (1986) The isolation and characterisation of six avian infectious bronchitis virus isolated in Morocco. *Avian Pathology* **15** : 93 - 105

ENDOMUNOZ, L. B. AND FARAGHER, J. T. (1989) A fluorescence test in allantoic cells for the detection of infectious bronchitis virus. *Australian Veterinary Journal* **66** : 338 - 340

EVANS, E. W., BEACH, F. G., MOORE, K. M., JACKWOOD, M. W., GLISSON, J. R. AND HARMON, B. G. (1995) Antimicrobial activity of chicken and turkey heterophil peptides CHP1, CHP2, THP1 and THP3. *Veterinary Microbiology* **47** : 295 - 303

FABRICANT, J. AND LEVINE, P. P. (1962) Experimental production of complicated chronic respiratory disease (air-sac disease) *Avian Diseases* **6** : 13 - 23

FAHEY, K. J., YORK, J. J. AND BAGUST, T. J. (1984) Larynotracheitis herpes virus infection in the chicken. II. The adoptive transfer of resistance with immune spleen cells. *Avian Pathology* **13** : 265 - 275

FITZGERALD, S. D., REED, W. M., FURUKAWA, A. M., ZIMELS, E. AND FUNG, L. (1995) Effect of T-lymphocyte depletion on the pathogenesis of marble spleen disease virus infection in ring-necked pheasants. *Avian Diseases* **39** : 68 - 73

FULTON, R. M., REED, M. AND DE NICOLA, D. B. (1990) Light microscopic and ultrastructural characterisaion of cells recovered by respiratory tract lavage of 2 and 6-week-old chickens. *Avian Diseases* **34** : 87 - 98

FULTON, R. M., REED, M. AND THACKER, H. L. (1993) Cellular response of the respiratory tract of chickens to infection with Massachusetts 41 and Australian T infectious bronchitis viruses. *Avian Diseases* **37** : 951 - 960

FULTON, R. M., REED, M., THACKER, H. L. AND DE NICOLA, D. B. (1996) Cyclophosphamide (Cytoxan)-induced haematologic alterations in specific-pathogen-free chickens. *Avian Diseases* **40** : 1 - 12.

GELB, J. JR. AND CLOUD, S. S. (1983) Effect of serial embryo passage of an Arkansas type avian infectious bronchitis virus isolate on clinical response, virus recovery and immunity. *Avian Diseases* **27** : 679 - 687

GELB, J., JR. AND KILLIAN, S. L. (1987) Serum antibody responses of chickens following sequential inoculations with different infectious bronchitis virus serotypes. *Avian Diseases* **31** : 513 - 522

GELB, J., LUNT, R. L., METZ, A. L. AND FRIES, P. A. (1991) Attenuation of avian infectious bronchitis virus by cold adaptation. *Avian Diseases* **35** : 847 - 853

GELB, J., PERKINS, JR., B. E., ROSENBERGER, J. K. AND ALLEN, P. H. (1981) Serologic and cross-protection studies with several infectious bronchitis virus isolates from Delmarva reared broiler chickens. *Avian Diseases* **25** : 655 - 666

GELB, J., ROSENBERGER, J. K., FRIES, P. A., CLOUD, S. S., ODOR, E. M., DOHMS, J. E. AND JAEGER, J. S. (1989) Protection afforded by infectious bronchitis virus vaccinated sentinel chickens raised in a commercial environment. *Avian Diseases* **33** : 764 - 769

GELB, J. JR., WOLFF, J. B. AND MORAN, C. A. (1991) Variant serotypes of infectious bronchitis virus isolated from commercial layer and broiler chickens. *Avian Diseases* **35** : 82 - 87

GILLETTE, K. G. (1973) Plaque formation by infectious bronchitis virus in chicken embryo kidney cell cultures. *Avian Diseases* **17** : 369 - 378

GILLETTE, K. G. (1974) Avian infectious bronchitis: demonstration of seum IgG and IgM neutralising anibody by sucrose densiy gradient centrifugation and mercaptoethanol reduction. *Avian Diseases* **18** : 515 - 525

GILLETTE, K. G. (1981) Local antibody responses to avian infectious bronchitis: virus neutralising antibody in tracheobronchial secretions. *Avian Diseases* **25** : 431 - 443

GOMEZ, L. AND RAGGI, L. G. (1974) Local immunity to avian infectious bronchitis virus in tracheal organ cultures. *Avian Diseases* **18** : 346 - 368

GOUGH, R. E. AND ALEXANDER, D. J. (1977) Comparison of serological tests for the measurement of primary immune response to avian infectious bronchitis virus vaccines. *Veterinary Microbiology* **2** : 289 - 301

GOUGH, R. E., COX, W. J., WINKLER, C. E., SHARP, M. W. AND SPACKMAN, D. (1996) Isolation and identification of infectious bronchitis virus from pheasants. *Veterinary Record* **138** : 208 - 209

GOUGH, R. E., RANDALL, C. J., DAGLESS, M., ALEXANDER, D. J., COX, W. J. AND PEARSON, D. (1992) A new strain of infectious bronchitis virus infecting domestic fowl in Great Britain. *Veterinary Record* **130** : 493 - 494

GUITTET, M., PICAULT, J. P., LE COQ, H., LAMANDE, J. AND BENNEJEAN, G. (1988) Strategy of protection of the layer against infectious bronchitis virus. *Proceedings of the Ist International Symposium on Infectious Bronchitis,* Rauischholzhausen, Germany, pp 298 - 307

HANSEN, M. B., ROSS, C. AND BERG, K. (1990) A sensitive antiviral neutralisation bioassay for measuring antibodies to interferons. *Journal of Immunological Methods* **127** : 241 - 248

HAWKES, R. A., DARBYSHIRE, J. H., PETERS, R. W., MOCKETT, A. P. A. AND CAVANAGH, D. (1983) Presence of viral antigens and antibody in the trachea of chickens infected with avian infectious bronchitis virus. *Avian Pathology* **12** : 331 - 340

HEATH, B. C. (1970) Chemical pathology of nephrosis induced by infectious bronchitis virus. *Avian Diseases* **14** : 95 - 106

HESSELINK, W. G., SCHRIER, C., JAGT, E. AND HOCKS, J. (1988) Detection of infectious bronchitis virus antigen by enzyme immunoassay: application for diagnostic purposes. *Proceedings of the Ist International Symposium on Infectious Bronchitis,* Rauischholzhausen, Germany, pp. 208 - 216

HILL, J. E., ROWLAND, G. N., LATIMER, K. S. AND BROWN, J. (1989) Effects of cyclosporine A on reovirus-infected broilers. *Avian Diseases* **33** : 86 - 92

HINZE, V., LOHR, J. E. AND KALETA, E. F. (1991) Infectious bronchitis virus strain differentiation attempts by cross-immunity studies in tracheal organ cultures derived from immunised chickens. *Proceedings of the IInd International Symposium.on Infectious Bronchitis* , Rauischholzhausen, Germany, pp. 73 - 86

HITCHNER, S. B., APPLETON, G. S. AND WINTERFIELD, R. W. (1964) Evaluation of the immune response to infectious bronchitis virus. *Avian Diseases* **8** : 153 - 162

HOFSTAD, M. S. (1981) Cross-immunity in chickens using seven isolates of avian infectious bronchitis virus. *Avian Diseases* **25** : 650 - 654

HOLMES, K. V. (1990) Coronaviridae and their replication. *Virology, Second edition, ed. B. N. Fields, D. M. Knipe et al., Raven Press, NewYork p 841*

HOLMES, H. C. AND DARBYSHIRE, J. H. (1978) Induction of chicken interferon by avian infectious bronchitis virus. *Research in Veterinary Science* **25** : 178 - 181

HOPKINS, S. R. (1974) Serological comparisons of strains of infectious bronchitis virus using plaque-purified isolants. *Avian Diseases* **18** : 231 - 239

HOPKINS, S. R. AND YODER, H. W. (1982) Influence of infectious bronchitis strains and vaccines on the incidence of *Mycoplasma synoviae* airsacculitis. *Avian Diseases* **26** : 741 - 752

HOVI, T., SUNI, J., HORTLING, L. AND VAHERI, A. (1978) Stimulation of chicken lymphocytes by T and B cell mitogens. *Cellular Immunology* **39** : 70 - 78

IGNJATOVIC, J. (1988) Epidemiology of infectious bronchitis in Australia. *Proceedings of the Ist International Symposium on Infectious Bronchitis,* Rauischholzhausen, Germany, pp. 84 - 88

IGNJATOVIC, J. AND GALLI. L. (1994) The S1 glycoprotein but not the N or M proteins of avian infectious bronchitis virus induces protection in vaccinated chickens. *Archives of Virology* **138** : 117 - 134

IGNJATOVIC, J. AND GALLI. L. (1995) Immune responses to structural proteins of avian infectious bronchitis virus. *Avian Pathology* **24** : 313 - 332

IGNJATOVIC, J. AND MCWATERS, P. G. (1991) Monoclonal antibodies to three structural proteins of avian infectious bronchitis virus: characterisation of epitopes and antigenic differentiation of Australian strains. *Journal of General Virology* **72** : 2915 - 2922

ISOBE, T. AND LILLEHOJ, H. S. (1993) Dexamethasone supresses T-cell mediated immunity and enhances disease susceptibility to *Eimeria mivati* infection. *Veterinary Immunology and Imunopathology* **39** : 431 - 446

JACKWOOD, M. W., MOO KWOON, H. AND HILT, D. A. (1992) Infectious bronchitis virus detection in the allantoic fluid using the polymerase chain reaction and a DNA probe. *Avian Diseases* **36** : 403 - 409

JANSE, E. M., VAN ROOZELAAR, D. AND KOCH, G. (1994) Leukocyte subpopulations in kidney and trachea of chickens infected with infectious bronchitis virus. *Avian Pathology* **23** : 513 - 523

JIA, W., KARACA, K., PARRISH, C. R. AND NAQI, S. A. (1995) A novel variant of avian infectious bronchitis virus resulting from recombination from three different strains. *Archives of Virology* **140** : 259 - 271

JOHNSON, R. B. AND MARQUARDT, W. W. (1975) The neutralising characteristics of strains of infectious bronchitis virus as measured by the constant-virus variable-serum method in chicken tracheal organ cultures. *Avian Diseases* **19** : 82 - 90

JONES, R. C. (1974) Nephrosis in laying chickens caused by Massachusetts-type of infectious bronchitis virus. *Veterinary Record* **95** : 319

JONES, R. C. AND AMBALI, A. G. (1987) Re-excretion of an enterotropic infectious bronchitis virus by hens at point of lay after experimental infection at day-old. *Veterinary Record* **120** : 617 - 619

JONES, R. C. AND JORDAN, F. T. W. (1970) The exposure of day-old chicks to infectious bronchitis and subsequent development of the oviduct. *Veterinary Record* **87** : 504 - 505

JONES, R. C. AND JORDAN, F. T. W. (1971) The site of replication of infectious bronchitis virus in the oviducts of experimentally infected hens. *Veterinary Record* **89** : 317 - 318

JONES, R. C. AND JORDAN, F. T. W. (1972) Persistence of the virus in the tissues and development of the oviduct in the fowl following infection at day-old with infectious bronchitis virus. *Research in Veterinary Science* **13** : 52 - 60

JONES, R. C., NAYLOR, C. J., BRADBURY, J. M., SAVAGE, C. E., WORTHINGTON, K. J. AND WILLIAMS, R. A. (1991) Isolation of a turkey rhinotracheitis-like virus from broiler breeder chickens in England. *Veterinary Record* **129** : 509 - 510

JONES, R. C., NAYLOR, C., AL-AFALEQ, A., WORTHINGTON, K. AND JONES, R. (1992) Effect of cyclophosphamide immunosuppression on the immunity of turkeys to viral rhinotracheitis. *Research in Veterinary Science* **53** : 38 - 41

JORDAN, F. T. W. AND NASSAR, T. J. (1973) The survival of infectious bronchitis virus in water. *Avian Pathology* **2** : 91 - 101

JUNGHERR, E. L., CHOMIAK, T. W. AND LUGINBUHL, R. E. (1956) Immunological difference in strains of infectious bronchitis virus. *60th meeting of the U.S. Livestock Sanitary Association.*

KANT, A., KOCH, G., VAN ROOZELAAR, D. J., KUSTERS, J. G., POELWIJK, F. A. J. AND VAN DER ZEILST, B. A M. (1992) Location of antigenic sites defined by neutralising monoclonal antibodies on the S1 avian infectious bronchitis virus glcopolypeptide. *Journal of General Virology* **73** : 591 - 596

KARACA, K. AND NAQI, S. A. (1993) A monoclonal antibody blocking ELISA to detect serotype-specific infectious bronchitis virus antibodies. *Veterinary Microbiology* **34** : 249 - 257

KARACA, K., NAQI, S. A. AND GELB, J. (1992) Production and characterisation of monoclonal antibodies to three infectious bronchitis virus serotypes. *Avian Diseases* **36** : 903 - 915

KARACA, K., NAQI, S. A., PALUKAITIS, P. AND LUCIO, B. (1990) Serological and molecular characterisation of three enteric isolates of infectious bronchitis virus of chickens. *Avian Diseases* **34** : 899 - 904

KINDE, H., DAFT, B. M., CASTRO, A. E., BICKFORD, A. A., GELB, J. AND REYNOLDS, B. (1991) Viral pathogenesis of a nephrotropic infectious bronchitis virus isolated from commercial pullets. *Avian Diseases* **35** : 415 - 421

KING, D. J. AND CAVANAGH, D. (1991) Infectious Bronchitis. in *Diseases of Poultry*. B. W. Calnek, J. H. Barnes, C. W. Beard, W. M. Reid and H. W. Yoder Jr. (Editors). Iowa State University Press, Ames, Iowa, USA

KING, D. J. AND HOPKINS, S. R. (1983) Evaluation of the haemagglutination-inhibition test for measuring the response of chickens to avian infectious bronchitis virus vaccination. *Avian Diseases* **27** : 100 - 112

KING, D. J. AND HOPKINS, S. R. (1984) Rapid serotyping of infectious bronchitis virus isolates with the haemagglutination-inhibition test. *Avian Diseases* **28** : 727 - 733

KLASING, K. C. AND PENG, R. K. (1987) Influence of cell sources, stimulating agents and incubation conditions on the release of interleukin -1 from chicken macrophages. *Developmental and Comparative Immunology* **11** : 385 - 393

KLASING, K. C. AND PENG, R. K. (1990) Monokine-like activity released from a chicken macrophage line. *Animal Biotechnology* **1** : 107 - 120

KLIEVE, A. V. AND CUMMING, R. B. (1988) Immunity and cross-protection to nephritis produced by Australian infectious bronchitis viruses used as vaccines. *Avian Pathology* **17** : 829 - 839

KLIEVE, A. V. AND CUMMING, R. B. (1990) Respiratory disease and immunity to challenge produced by Australian strains of infectious bronchitis virus. *Avian Pathology* **19** : 305 - 312

KOCH, G., HARTOG, L., KANT, A. AND VAN ROOZELAAR, D. J. (1990) Antigenic domains on the peplomer proteins of avian infectious bronchitis virus: correlation with biological functions. *Journal of General Virology* **71** : 1929 - 1935

KOGUT, M. H., TELLEZ, G., HARGIS, B. M., CORRIER, D. E. AND DELOACH, J. R. (1993) The effect of 5-fluorouracil treatment of chicks: a cell depletion model for the study of avian polymorphonuclear leukocytes and natural host defences. *Poultry Science* **72** : 1873 - 1880

KOTTIER, S. A., CAVANAGH, D. AND BRITTON, P. (1995) Experimental evidence of recombination in coronavirus infectious bronchitis virus. *Virology* **213** : 569 - 580

KRONER, H., SCHLIEPHAKE, A., WINTER, J., ZIMPRICH, F., LASSMANN, H., SEDWICK, J. AND WEGE, H. (1991) Nucleocapsid or spike protein-specific CD4 T-lymphocytes protect against coronavirus-induced encephalomyelitis in the absence of CD8 T-cells. *Journal of Immunology* **147** : 2317 - 2323

KURZMAN, I. D., SHI, F. AND MACEWEN, E. G. (1993) *In-vitro* and *in-vivo* canine mononuclear cell production of tumour necrosis factor induced by muramyl peptides and lipopolysaccharide. *Veterinary Immunology and Immunopathology* **38** : 45 - 56

KUSTERS, J. G., JAGER, E. J., NEISTERS, H. G. M. AND VAN DER ZEIJST, B. A. M. (1990) Sequence evidence for RNA recombination in field isolates of avian corona virus, infectious bronchitis virus. *Vaccine* **8** : 605 - 608

KUSTERS, J. G., NIESTERS, H. G. M., BLEUMIN-PLUYM, N. M. C., DAVELAAR, F. G., HORZINEK, M. C. AND VAN DER ZEIJST, B. A. M. (1987) Molecular epidemiology of infectious bronchitis virus in the Netherlands. *Journal of General Virology* **68** : 343- 352

KUSTERS, J. G., NIESTERS, H. G. M., LENSTRA, J. A., HORZINEK, M. C. AND VAN DER ZEIJST, B. A. M. (1989) Phylogeny of antigenic variants of avian corona virus, infectious bronchitis virus. *Virology* **169** : 217 - 221

KWON, H. M. AND JACKWOOD, M. W. (1995) Molecular cloning and sequence comparison of the S1 glycoprotein of the Gray and JMK strains of avian infectious bronchitis virus. *Virus Genes* **9** : 219 - 229

KWON, H. M., JACKWOOD, M. W., BROWN, T. P. AND HILT, D. A. (1993a) Polymerase chain reaction and a biotin-labeled DNA probe for detection of infectious bronchitis virus in chickens. *Avian Diseases* **37** : 149 - 156

KWON, H. M., JACKWOOD, M. W. AND GELB, J. (1993b) Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. *Avian Diseases* **37** : 194 - 202

LAMBRECHTS, C., PENSAERT, M. AND DUCATELLE, R. (1991) Comparative study of the infectivity of Belgian NIBV isolates for the kidney after intravenous inoculation in chickens. *Proceedings of the IInd International Symposium on Infectious bronchitis.*, Rauischholzhausen, Germany, pp. 113 - 119

LAMBRECHTS, C., PENSAERT, M. AND DUCATELLE, R. (1993) Challenge experiments to evaluate cross-protection induced at the trachea and kidney level by vaccine strains and Belgian nephropathogenic isolates of avian infectious bronchitis virus. *Avian Pathology* **22** : 577 - 590

LAROSE, R. N. AND VAN ROEKEL, H. (1961) The effects of rapid embryo passage upon infectious bronchitis virus. *Avian Diseases* **5** : 157 - 168

LASHGARI, M. S. AND NEWMAN, J. A. (1984) Serological comparison and antigenic relationships of seven serotypes of infectious bronchitis virus using the haemagglutination-inhibition test. *Avian Diseases* **28** : 436 - 443

LEE, L. F. (1978) Chicken lymphocyte stimulation by mitogens: a microassay with whole blood cultures. *Avian Diseases* **22** : 296 - 307

LEE, L. F., SHARMA, J. M., NAZERIAN, K. AND WITTER, R. L. (1978) Suppression of mitogen induced proliferation of normal spleen cells by macrophages from chickens inoculated with Marek's disease virus. *Journal of Immunology* **120** : 1554- 1559

LILLEHOJ, H. S. (1987) Effects of immunosuppression on avian coccidiosis: cyclosporin A but not hormonal bursectomy abrogates host protective immunity. *Infection and Immunity* **55** : 1616 - 1621

LILLEHOJ, H. S., LILLEHOJ, E. P., WEINSTOCK, D. AND SCHAT, K. (1988) Functional and biochemical characterisation of chicken T-lymphocyte antigens. *European Journal of Immunology* **18** : 2059 - 2065

LIN, Z., KATO, A., KUDOU, Y. AND UEDA, S. (1991a) A new typing method for the avian infectious bronchitis virus using polymerase chain reaction and restriction enzyme fragment length polymorphism. *Archives of Virology* **116** : 19 - 31

LIN, Z., KATO, A., KUDOU, Y., UMEDA, K. AND UEDA, S. (1991b) Typing of recent infectious bronchitis virus isolates causing nephritis in chickens. *Archives of Virology* **120** : 145 - 149

LOHR, J. E. (1988) Differentiation of IBV strains. *Proceedings of the Ist International Symposium on Infectious Bronchitis,* Rauischholzhausen, Germany, pp. 199 - 207

LOHR, J. E., HINZE, V. AND KALETA, E. F. (1991) Immunological relationship between the New Zealand A and the Australian T strains of infectious bronchitis virus as measured by cross-immunisation tests in tracheal organ cultures from immunised birds. *New Zealand Veterinary Journal* **39** : 113 - 114

LOMNICZI, B. (1974) Interferon induction by different strains of infectious bronchitis virus. *Acta Veterinarica Hungarica* **24** : 199 - 204

LUCAS, A. M. AND JAMROZ, C. (1961) Atlas of Avian Haematology. Agricultural monograph 25. USDA, Washington, DC.

LUCIO, B. AND FABRICANT, J. (1990) Tissue tropism of three cloacal isolates and Massachusetts strain of infectious bronchitis virus. *Avian Diseases* **26** : 508 - 519

LUCIO, B. AND HITCHNER, S. B. (1970) Differentiation and detection of infectious bronchitis virus subtypes by immunofluoescence. *Avian Diseases* **14** : 9 - 24

LUKERT, P. D. (1965) Comparative sensitivities of embryonating chickens eggs and primary chicken embryo kidney and liver cell cultures to infectious bronchitis virus. *Avian Diseases* **9** : 308 - 316

LUTTICKEN, D., RIJKE, E. O., LOEFFEN, T. AND HESSELINK, W. G. (1988) Aspects of local immune response to IBV. *Proceedings of the Ist international Symposium on Infectious Bronchitis,* Rauischholzhausen, Germany, pp. 173 - 181

MACDONALD, J. W. AND MCMARTIN, D. A. (1976) Observations on the effects of the H52 and H120 vaccine strains of infectious bronchitis virus in the domestic fowl. *Avian Pathology* **5** : 157 - 173

MACDONALD, J. W., RANDALL, C. J. AND MCMARTIN, D. A. (1980) An inverse age resistance of chicken kidneys to infectious bronchitis virus. *Avian Pathology* **9** : 245 - 259

MACDONALD, J. W., RANDALL, C. J., MCMARTIN, D. A., DAGLESS, M. D. AND GAZDZINSKI, P. (1981) Active and passive immunisation against nephritis induced by avian infectious bronchitis virus. *Avian Pathology* **10** : 121 - 129

MACPHERSON, I. AND FEEST, A. (1978) Some observations on the value of infecious bronchitis haemagglutination-inhibition test in the field. *Avian Pathology* **7** : 337 - 347

MANNEL, D. N., MOORE, R. N. AND MERGEN, S. E. (1980) Macrophages as a source of tumouricidal activity (tumour-necrotising factor). *Infection and Immunity* **30** : 523 - 530

MARQUARDT, W.W., KADAVIL, S. K. AND SNYDER, D. B. (1982) Comparison of ciliary activity and virus recovery from tracheas of chickens and humoral immunity after inoculation with serotypes of avian infectious bronchitis virus. *Avian Diseases* **26** : 828 - 834

MARQUARDT, W.W., SNYDER, D. B. AND SCHLOTTHOBER, B. A. (1981) Detection and quantification of antibodies to infectious bronchitis virus by enzyme linked immunosorbent assay. *Avian Diseases* **25** : 713 - 722

MARTINS, N. R. D., MOCKETT, A. P. A., BARRETT, A. D. T. AND COOK, J. K. A. (1991) IgM responses in chicken serum to live and inactivated infectious bronchitis virus vaccines. *Avian Diseases* **35** : 470 - 475

MCDOUGALL, J. S. (1968) Infectious bronchitis in laying fowls: its effect upon egg production and subsequent egg quality. *Veterinary Record* **83** : 84 - 86

MCFERRAN, J. B., CLARKE, J. K. AND URRAN, W. L. (1971) The application of negative contrast electron microscopy to routine veterinary virus diagnosis. *Research in Veterinary Science* **12** : 253 - 257

MCGEE, Z., JOHNSON, A. AND TAYLOR-ROBINSON, D. (1976) Human fallopian tubes in organ culture: preparation, maintainance and quantitation of damage by pathogenic organisms. *Infection and Immunity* **13** : 608 - 618

MCINTOSH, K., DEES, J. H., BECKER, W. B., KAPIKIAN, A. Z. AND CHANOCK, R. M. (1967) Recovery in tracheal organ cultures of novel viruses from patients with respiratory disease. *Proceedings of the National Academy of Sciences of the USA* **57** : 933 - 940

MCINTYRE, K. W., UNIWSKY, J., DELORENZO, W. AND BENJAMIN, W. (1989) Enhancement of antibacterial resistance of neutropaenic bone marrow-suppressed mice by interleukin-1. *Infection and Immunity* **57** : 48 - 54

MCMARTIN, D. A. (1968) The pathogenicity of an infectious bronchitis virus for laying hens, with observations on pathogenesis. *British Veterinary Journal* **124** : 576 - 581

MCMARTIN, D. A. (1993) Inectious bronchitis. In Virus Infections of Vertebrates. *Virus infections of birds.* Ed. McFerran, J. B. and McNulty, M. S., M. S. Elsevier Science Publishers. Volume **4**. pp. 249 - 275

MEULEMANS, G., CARLIER, M. C., GONZE, M., PETIT, P. AND VANDENBROUCK, M. (1987) Incidence, characterisation and prophylaxis of nephropathogenic avian infectious bronchitis virus. *Veterinary Record* **120** : 205

MIGGIANO, V., NORTH, M., BUDER, A. AND PINK, J. R. L. (1976) Genetic control of the response of chicken leucocytes to a T-cell mitogen. *Nature* **263** : 61 - 63

MILLER, L. AND QURESHI, M. A. (1992) Comparison of heat-shock induced and lipopolysaccharide induced protein changes and tumouricidal activity in a chicken mononuclear cell line. *Poultry Science* **71** : 979 - 987

MIMMS, C. A. (1988) Immunobiology and pathogenesis of persistent virus infections. In: *Immunobiology and pathogenesis of persistent virus infections.* C. Lopez, Ed. American Society for Microbiology, Washington D.C. pp. 3 - 17

MOCKETT, A. P. A. (1986) Studies on the humoral immune system of the chicken and its response to avian infectious bronchitis virus infection. *Ph.D. thesis*, University of Liverpool.

MOCKETT, A. P. A., CAVANAGH, D. AND BROWN, T. D. K. (1984) Monoclonal antibodies to the S1 spike and membrane proteins of avian infectious bronchitis corona virus strain Massachusetts M41. *Journal of General Virology* **65** : 2281 - 2286

MOCKETT, A. P. A. AND COOK, J. K. A. (1986) The detection of specific IgM to infectious bronchitis virus in chicken serum using an ELISA. *Avian Pathology* **15** : 437 - 446

MOCKETT, A. P. A., COOK, J. K. A. AND HUGGINS, M. B. (1987) Maternally-derived antibody to infectious bronchitis virus: its detection in chick trachea and serum and its role in protection. *Avian Pathology* **16** : 407 - 416

MOCKETT, A. P. A. AND DARBYSHIRE, J. H. (1981) Comparative studies with an enzyme linked immunosorbent assay for antibodies to avian infectious bronchtis virus. *Avian Pathology* **10** : 1 - 10

MONREAL, G., BAUER, H. J. AND WIEGMANN, J. (1985) Comparison of enzyme linked immunosorbent assay (ELISA), haemagglutination-inhibition test and agar-gel precipitation test for detection of antibodies to avian infectious bronchitis virus. *Avian Pathology* **14**: 421 - 434

MONTGOMERY, R. D., MASLIN, W. R., BOYLE, C. R., PLEDSER, T. AND WU, CHING-CHING (1991) Effects of an Arkansas strain of infectious bronchitis vaccine on the head-associated lymphoid tissue (HALT). *Avian Diseases* **35**: 302 - 307

MONTGOMERY, R. D., MASLIN, W. R., MAGEE, D. L. AND BOYLE, C. R. (1994) Effects of modified live infectious bronchitis virus vaccines on the head-associated lymphoid tissue. *Avian Diseases* **38**: 847 - 856

MORLEY, A. J. AND THOMPSON, D. K. (1984) Swollen head syndrome in broiler chickens. *Avian Diseases* **28**: 238 - 243

MOSMANN, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65**: 55 - 63

MOSMANN, T. R., CHERWINSKI, H., BOND, M. W., GIEDLIN, M. A. AND COFFMAN, R. J. (1986) Two types of murine helper T-cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of Immunology* **136**: 2348 - 2357

MUNEER, M. A., HALVORSON, D. A., SIVANANDAN, V., NEWMAN, J. A. AND COON, C. N. (1986) Effects of infectious bronchitis (Arkansas strain) on laying chickens. *Avian Diseases* **30**: 644 - 647

MUNEER, M. A., NEWMAN, J. A., HALVORSON, D. A., SIVANANDAN, V. AND COON, C. N. (1987) Effects of avian infectious bronchitis virus (Arkansas strain) on vaccinated laying chickens. *Avian Diseases* **31**: 820 - 828

MUNEER, M. A., NEWMAN, J. A., HALVORSON, D. A., SIVANANDAN, V., NAGARAJA, K. V. AND COON, C. N. (1988) Efficacy of infectious bronchitis virus vaccines against heterologous challenge. *Research in Veterinary Science* **45**: 22 - 27

NAGANO, H., HASHIMOTO, H., TANAKA, Y. AND FUJISAKI, Y. (1993) Dot blot hybridisation using digoxigenin-labeled cDNA probe complementary to the S1 gene of avian infectious bronchitis virus permits discrimination between virus strains. *Journal of Veterinary Medical Science* **55** : 735 - 738

NAKAMURA, K., COOK, J. K. A., FRAZIER, J. A. AND NARITA, M. (1992) *Escherichia coli* multiplication and lesions in the respiratory tract of chickens inoculated with infectious bronchitis virus and/or *Escherichia coli*. *Avian Diseases* **36** : 881 - 890

NAKAMURA, K., COOK, J. K. A., OTSUKI, K., HUGGINS, M. B. AND FRAZIER, J. A. (1991) Comparative study of respiratory lesions in two chicken lines of different susceptibility, infected with infectious bronchitis virus: histology, ultrastructure and immunochemistry. *Avian Pathology* **20** : 245 - 261

NAKAMURA, K., IMAI, K. AND TANIMURA, N. (1996) Comparison of the effects of infectious bronchitis and infectious laryngotracheitis on the chicken respiratory tract. *Journal of Comparative Pathology* **114** : 11 - 21

NAKAMURA, K., UEDA, H., TANIMURA, T. AND NOGUCHI, K. (1994) Effect of mixed live vaccine (ND and IB) and *Mycoplasma gallisepticum* on the chicken respiratory tract and on *Escherichia coli* infection. *Journal of Comparative Pathology* **111** : 33 - 42

NAQI, S. A. (1990) A monoclonal antibody based immunoperoxidase procedure for rapid detection of infectious bronchitis virus in infected tissues. *Avian Diseases* **34** : 893 - 898

NAQI, S. A., KARACA, K. AND BAUMAN, B. (1993) A monoclonal antibody based antigen capture enzyme linked immunosorbent assay for identification of infectious bronchitis virus serotypes. *Avian Pathology* **22** : 555 - 564

NATT, M. P. AND HERRICK, C. A. (1952) A new blood diluent for counting the erythrocytes and leukocytes of chickens. *Poultry Science* **31** : 735 - 738

NAYLOR, C. J. AND JONES, R. C. (1994) Demonstration of a virulent subpopulation in a prototype live attenuated turkey rhinotracheitis vaccine. *Vaccine* **12** : 1225 - 1230

NIESTERS, H. G. M., BLEUMINKPLUYM, N. M. C., OSTERHAUS, A. D. M. E., HORZINEK, M. C. AND VANDERZEIJST, B. A. M. (1987) Epitopes on the peplomer protein of infectious bronchitis virus strain M41 as defined by monoclonal antibodies. *Virology* **161** : 511 - 519

NOWAK, J. S., KAI, O., PECK, R. AND FRANKLIN, R. M. (1982) The effects of cyclosporine A on the chicken immune system. *European Journal of Immunology* **12** : 867 - 876

NYSKA, A., SKOLNICK, M., ZIV, G. AND GULKAROV, A. (1994) Correlation of injection site damage and creatine kinase activity in turkeys following intramuscular and subcutaneous adminstration of norfloxacin nicotinate. *Avian Pathology* **23** : 671 - 682

ORTALDO, J. R., BONNARD, G. D., KIND, P. D. AND HERBERMEN, R. (1979) Cytotoxicity of cultured lymphocytes: characteristics of effector cells and specificity of toxicity. *Journal of Immunology* **122** : 1489 - 1494

OSSERMAN, E. F. AND LAWLOR, D. P. (1966) Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. *Journal of Experimental Medicine* **124** : 921 - 929

OTSUKI, K., DAVISON, T. F. AND COOK, J. K. A. (1991) Interferon induction by avian infectious bronchitis virus. *Proceedings of the IInd International Symposium on Infectious Bronchitis,* Rauischholzhausen, Germany, pp. 137 - 142

OTSUKI, K., HUGGINS, M. B. AND COOK, J. K. A. (1990) Comparison of the susceptibility to avian infectious bronchitis virus infection of two inbred lines of white leghorn chickens. *Avian Pathology* **19** : 467 - 475

OTSUKI, K., MAEDA, J., YAMAMOTO, H. AND TSUBOKURA, M. (1979b) Studies on avian infectious bronchitis virus (IBV). III. Interferon induction by and sensitivity to interferon of IBV. *Archives of Virology* **60** : 249 - 255

OTSUKI, K., NAKAMURA, T., KAWAOKA, Y. AND TSUBOKURA, M. (1988) Interferon induction by several strains of avian infectious bronchitis virus, a corona virus, in chickens. *Acta Virologica* **32** : 55 - 59

OTSUKI, K., YAMAMOTO, H. AND TSUBOKURA, M. (1979a) Studies on avian infectious bronchitis virus (IBV). I. Resistance of IBV to chemical and physical treatments. *Archives of Virology* **60** : 25 - 32

OWEN, R. L., COWEN, B. S., HATTEL, A. L., NAQI, S. A. AND WILSON, R. A. (1991) Detection of viral antigen following exposure of one-day old chickens to the Holland-52 strain of infectious bronchitis virus. *Avian Pathology* **20** : 663 - 673

PARSONS, D., ELLIS, M. M., CAVANAGH, D. AND COOK, J. K. A. (1992) Characterisation of an infectious bronchitis virus isolated from vaccinated broiler breeder flocks. *Veterinary Record* **131** : 408 - 411

PATTERSON, S. AND BINGHAM, R. W. (1976) Electron microscope observations on the entry of avian infectious bronchitis virus into susceptible cells. *Archives of Virology* **52** : 191 - 200

PATTISON, M., GOUGH, R. E. AND DAWSON, P. S. (1971) The effect of live infectious bronchitis vaccine on the course of infectious laryngotracheitis. *Veterinary Record* 75 : 643 - 644

PENSAERT, M. AND LAMBRECHTS, C. (1994) Vaccination of chickens against a Belgian nephropathogenic strain of infectious bronchitis virus B1648 using attenuated homologous and heterologous strains. *Avian Pathology* **23** : 631 - 641

PETERS, R. W., DARBYSHIRE, J. H. AND COOK, J. K. A. (1979) The susceptibility of chicken kidney and oviduct organ cultures to a vaccine strain of infectious bronchitis virus. *Research in Veterinary Science* **26** : 38 - 40

PICAULT, J. P., DROUIN, P., GUITTET, M., BENNEJEAN, G., PROTUIS, J., L'HOSPITALIEN, R., GILLET, J. P., LAMANDE, J. AND BUCHELIEN, A. (1986) Isolation, characterisation and preliminary cross-protection studies with a new pathogenic avian infectious bronchitis virus (strain PL-84084). *Avian Pathology* **15** : 367 - 383

PICAULT, J. P., DROUIN, P., TOUX, J. Y., GUITTET, M. AND BENNEJEAN, G (1991) Infectious bronchitis in France: Epidemiological situation and prophylactic practices. *Proceedings of the IInd International Symposium on Infectious Bronchitis*, Rauischholzhausen, Germany, pp. 3 - 22

PICAULT, J. P., GIRAUD, P., DROUIN, P., GUITTET, M., BENNEJEAN, G., LAMANDE, J., TOQUIN, D. AND GUEGUEN, C. (1987) Isolation of a turkey rhinotracheitis-like virus from chickens with swollen head syndrome. *Veterinary Record* **121** : 135

POHL, R. (1974) The histopathogenesis of the nephrosis-nephritis syndrome. *Avian Pathology* **3** : 1 - 13

PRADHAN, H. K., MOHANTY, G. C. AND RAJYA, B. S. (1984) Comparative sensitivities of oviduct and tracheal organ cultures and chicken embryo kidney cultures to infectious bronchitis virus. *Avian Diseases* **27** : 594 - 601

PROWSE, S. J. AND PALLISTER, J. (1989) Interferon release as a measure of the T-cell response to coccidial antigens in chickens. *Avian Pathology* **18** : 619 - 630

PURCELL, D. A. AND CLARKE, J. D. (1972) The replication of infectious bronchitis virus in fowl trachea. *Arch. Gesamte Virusforsch* **39** : 248 - 256

PURCELL, D. A. AND MCFERRAN, J. B. (1972) The histopathology of infectious bronchitis in the domestic fowl. *Research in Veterinary Science* **13** : 116 - 122

PURCELL, D. A., THAM, V. L. AND SURMAN, P. G. (1976) The histopathology of infectious bronchitis in fowls infected with a nephrotropic T strain of virus. *Australian Veterinary Journal* **52** : 85 - 91

PURCHASE, H. G., CUNNINGHAM, C. H. AND BURMESTER, B. R. (1966) Identification and epizootiology of infectious bronchitis in a closed flock. *Avian Diseases* **10** : 111 - 121

QURESHI, M. A., PETITTE, J. N., LASTER, S. M. AND DIETERT, R. R. (1993) Avian macrophages: contribution to cellular microenvironment and changes in effector functions following activation. *Poultry Science* **72** : 1280 - 1284

RAGGI, L. G. AND GOMEZ, L. (1975) Two new isolants of infectious bronchitis with polyvalent immunogenicity. *Avian Diseases* **19** : 323 - 333

RAGGI, L. G. AND LEE, G. G. (1965) Lack of correlation between infectivity, serologic response and challenge results in immunisation with an avian infectious bronchitis vaccine. *Journal of Immunology* **94** : 538 - 543

RAGGI, L. G. AND PIGNATTELLI, P. (1975) Identification of infectious bronchitis virus by interference with the B-1 isolant of Newcastle disease virus: waxing and waning of interference. *Avian Diseases* **19** : 334 - 342

RATANASETHAKUL, C. AND CUMMING, R. B. (1983a) Immune response of chickens to various routes of administration of Australian infectious bronchitis vaccine. *Australian Veterinary Journal* **60** : 214 - 216

RATANASETHAKUL, C. AND CUMMING, R. B. (1983b) Effect of environmental temperature on the mortality in vaccinated chickens after challenge with Australian infectious bronchitis virus. *Australian Veterinary Journal* **60** : 254 - 256

REDIG, P. T., DUNNETTE, J. L., MAURO, L., SIVANANDAN, V. AND MARKHAM, F. (1985) The *in-vitro* response of turkey lymphocytes to steroid hormones. *Avian Diseases* **29** : 373 - 383

REED, L. J. AND MUENCH, H. (1938) A simple method of estimating fifty percent endpoints. *American Journal of Hygiene* **27** : 493 - 497

ROSSACK, R. E., GUERRANT, R. L., DENSEN, P., SCHADELIN, J. AND MANDELL, G. L. (1981) Diminished oxidative metabolism after phagocytosis of virulent *Salmonella typhi. Infection and Immunity* **31** : 674 - 678

RUSSELL, P. H. (1993) Newcastle disease virus : virus replication in the Harderian gland stimulates lachrymal IgA; the yolk sac provides early lachrymal IgG. *Veterinary Immunology and Immunopathology* **37** : 157 - 163

SADASIV, E. C., YEH, T. T. AND CHANG, P. W. (1991) Protein iso-electric point (PI) alteration related to strain variation of infectius bronchitis virus, an avian corona virus. *Journal of Virological Methods* **33** : 115 - 125

SAIF, L. J. (1993) Coronavirus immunogens. *Veterinary Microbiology* **37** : 285 - 297

SAPATS, S. I., ASHTON, F., WRIGHT, P. J. AND IGNJATOVIC, J. (1996) Sequence analysis of the S1 glycoprotein of infectious bronchitis viruses - identification of a novel genotypic group in Australia. *Journal of General Virology* **77** : 413 - 418

SCHALK, A. E. AND HAWN, M. C. (1931) An apparently new respiratory disease of baby chicks. *Journal of American Veterinary Medical Association* **78** : 413 - 422

SCHREIBER, S. L. AND CRABTREE, G. R. (1992) The mechanism of action of cyclosporin A and FK 506. *Immunology Today* **13** : 136 - 141

SCHULTZ, R. D. (1982) Assays of cellular immunity. *Journal of American Veterinary Medical Association* **181**: 1169 - 1176

SEVOIAN, M. AND LEVINE, P. P. (1957) Effects of infectious bronchitis on the reproductive tract, egg production and egg quality of laying chickens. *Avian Diseases* **1** : 136 - 164

SHARMA, J. M. AND BELZER, S. W. (1992) Blastogenic response of whole blood cells of turkeys to a T-cell mitogen. *Developmental and Comparative Immunology* **16** : 77 - 84

SHARMA, J. M. AND LEE, L. F. (1983) Effect of infectious bursal disease on natural killer cell activity and mitogenic response of chicken lymphoid cells. The role of adherent cells in cellular immune supression. *Infection and Immunity* **40** : 747 - 754

SHARMA, R. K. AND WANG, J. H. (1986) Preparation and assay of the $Ca^{2+}$-dependent modular protein. *Advances in Cyclic Nucleotide Research* **10** : 187 - 198

SHAW, K., BRITTON, P. AND CAVANAGH, D. (1996) Sequence of the spike protein of the Belgian B1648 isolate of nephropathogenic infectious bronchitis virus. *Avian Pathology* **25** : 607 - 611

SHER, A. AND COFFMAN, R. L. (1992) Regulation of immunity to parasites by T-cells and T-cell derived cytokines. *Annual Review of Immunology* **10** : 385 - 409

SHIRAI, J., MAEDA, M., FUJII, M. AND KUNIYOSHI, S. (1993) Swollen head syndrome is not associated with turkey rhinotracheitis virus. *Veterinary Record* **132** : 41 - 42

SILLER, W. G. AND CUMMING, R. B. (1974) The histopathology of an interstitial nephritis in the fowl produced experimentally with infectious bronchitis virus. *Journal of Pathology* **114** : 163 - 173

SLOCOMBE, R. F., MALARK, J., INGERSOLL, R., DERKSEN, D. AND ROBINSON, N. (1985) Importance of neutrophils in the pathogenesis of acute pneumonic pasteurellosis in calves. *American Journal of Veterinary Research* **46** : 2253 - 2258

SMITH, J. A. (1994) Neutrophils, host defence and inflammation: a double edged sword. *Journal of Leukocyte Biology* **56** : 672 - 686

SMITH, H.W., COOK, J. K. A. AND PARCELL, Z. E. (1985) The experimental infection of chickens with mixtures of infectious bronchitis virus and *Escherichia coli*. *Journal of General Virology* **66** : 777 - 786

SMITS, B., ROSENDAL, S., RUHNKE, H. L., PLANTE, C., O'BRIEN, P. J. AND MILLER, R. B. (1994) Effects of *Ureaplasma diversum* on bovine oviductal explants: Quantitative measurement using a calmodulin assay. *Canadian Journal of Veterinary Research* **58** : 114 - 121

SNYDER, D. B., MARQUARDT, W. W. AND KADAVIL, S. K. (1983) Ciliary activity - a criterion for associating resistance to infectious bronchitis virus infection with ELISA antibody titre. *Avain Diseases* **27** : 485 - 490

SPACKMAN, D. AND CAMERON, I. D. R. (1983) Isolation of infectious bronchitis virus from pheasants. *Veterinary Record* **113** : 354 - 355

STADTLANDER, C. T. K., WATSON, H. L., SIMECKA, J. W. AND CASSELL, G. H. (1991) Cytopathic effects of *Mycoplasma pulmonis in-vivo* and *in-vitro*. *Infection and Immunity* **59** : 4201 - 4211

STOHLMAN, S. A., MATSUSHIMA, G. K., CASTEEL, N. AND WEINER, L. P. (1986) *In-vivo* effects of corona virus specific T-cell clones: DTH inducer cells prevent a lethal infection but do not inhibit viral replication. *Journal of Immunology* **136** : 3052 - 3056

SUNG, Y.-J., HOTCHKISS, J. H., AUSTIC, R. E. AND DIETERT, R. R. (1991) L-arginine dependent production of a reactive nitrogen intermediate by macrophages of a uricotelic species. *Journal of Leukocyte Biology* **51** : 49 - 56

SURESH, M. AND SHARMA, J. M. (1995) Haemorrhagic enteritis virus induced changes in the lymphocyte subpopulations in turkeys and the effect of experimental immunodeficiency on viral pathogenesis. *Veterinary Immunology and Immunopathology* **45** : 139 - 150

SURVASHE, B. D., AITKEN, I. D. AND POWELL, J. R. (1979) The response of the Harderian gland of the fowl to antigen given by the ocular route. I. Histologic changes. *Avian Pathology* **8** : 77 - 93

TALEBI, A., TORGERSON, P. R. AND MULCAHY, G. (1995) Optimal conditions for measurement of blastogenic response of chickens to concanavalin A in whole blood assays. *Veterinary Immunology and Immunopathlogy* **46** : 293 - 301

TIMMS, L. M., BRACEWELL, C. D. AND ALEXANDER, D. J. (1980) Cell mediated and humoral immune response in chickens infected with avian infectious bronchitis. *British Veterinary Journal* **136** : 349 - 354

TIMMS, L. M. AND BRACEWELL, C. D. (1981) Cell mediated and humoral immune response of chickens to live infectious bronchitis vaccines. *Research in Veterinary Science* **31** : 182 - 189

TIMMS, L. M. AND BRACEWELL, C. D. (1983) Cell mediated and humoral immune response of chickens to inactivated oil-emulsion infectious bronchitis vaccines. *Research in Veterinary Science* **34** : 224 - 230

TOIVANEN, P. AND TOIVANEN, A. (1973) Selective activation of chicken T lymphocytes by concanavalin A . *Journal of Immunology* **111** : 1602 - 1603

TORO, H. AND FERNANDEZ, I. (1994) Avian infectious bronchitis - specific lachrymal IgA level and resistance to challenge. *Journal of Veterinary Medicine B series* **41** : 467 - 472

TORO, H., GODOY, V., LARENAS, J., REYES, E. AND KALETA, E. F. (1996) Avian infectious bronchitis - viral persistence in the Harderian gland and histological changes after eye-drop vaccination. *Avian Diseases* **40** : 114 - 120

TORO, H., HIDALGO, H. AND COLLINGWOODSELBY, A. N. (1994) Lachrymal antibody response of specific-pathogen-free chickens and chickens with maternal immunity after infectious bronchitis virus vaccination. *Preventive Veterinary Medicine* **18** : 267 - 274

TORO, H., LAVAND, P. AND VALLEJOS, P. AND FERREIRA, A. (1993) Transfer of IgG from serum to lachrymal fluid in chickens. *Avian Diseases* **37** : 60 - 66

TORO, H., SCHEMERA, B. AND KALETA, E. F. (1987) Serological differentiation of avian infectious bronchitis field isolates using an enzyme immunoassay: presence of Dutch strains in West Germany. *Avian Diseases* **31** : 187 - 192

TUMPEY, T. M., SHUN-HUA CHEN, OAKES, J. E. AND LAUSCH, R. N. (1996) Neutrophil-mediated suppression of virus replication after Herpes simplex virus type 1 infection of the murine cornea. *Journal of Virology* **70** : 898 - 904

VAINIO, O. AND RATCLIFFE, M. J. H. (1984) Proliferation of chicken peripheral blood leukocytes in response to pokeweed mitogen is macrophage-dependent. *Cellular Immunology* **85** : 235 - 243

VAINIO, O., VEROMAA, T., EEROLA, E., TOIVANEN, P. AND RATCLIFFE, M. J. H. (1988) Antigen-presenting cell- T-cell interaction in the chicken is MHC class II antigen restricted. *Journal of Immunology* **140** : 2864 - 2868

VAN DER MEER, J. W. M., BARZA, M., WLOFF, S. M. AND DINARELLO, C. A. (1988) A low dose of recombinant interleukin-1 protects granulocytopaenic mice from lethal Gram negative infection. *Proceedings of National Academy of Sciences.* **85** : 1620 - 1623

VERDUGO, P., RAESS, B. V. AND VILLALON, M. (1983) The role of calmodulin in the regulation of ciliary movement in mammalian somatic cells. *Journal of Submicroscopic Cytology and Pathology* **15** : 95 - 96

VON BULOW, V. AND KLASEN, A. (1983) Effects of avian viruses on cultured chicken bone-marrow derived macrophages. *Avian Pathology* **12** : 179 - 198

WADEY, C. N. AND FARAGHER, J. T. (1981) Australian infectious bronchitis viruses: identification of nine subtypes by a neutralisation test. *Research in Veterinary Science* **30** : 70 - 74

WADEY, C. N. AND WESTAWAY, E. G. (1981) Structural proteins and glycoproteins of infectious bronchitis virus particles labelled during growth in chick embryo cells. *Intervirology* **15** : 19 - 27

WAINRIGHT, P. O., VILLEGAS, P., BRUGH, M. AND LUKERT, P. D. (1989) Characterisation of infectious bronchitis virus using monoclonal antibodies. *Avian Diseases* **33** : 482 - 490

WAKENELL, P. S. AND SHARMA, J. M. (1986) Chicken embryonal vaccination with avian infectious bronchitis virus. *Americal Journal of Veterinary Research* **47** : 933 - 938

WAKENELL, P. S., SHARMA, J. M. AND SLOCOMBE, R. F. (1995) Embryo vaccination of chickens with infectious bronchitis virus - histologic and ultrastructural lesion response and immunological response to vaccination. *Avian Diseases* **39** : 752 - 765

WANG, L., JUNKER, D., HOCK, L., EBIARY, E. AND COLLISSON, E. W. (1994) Evolutionary implications of genetic variations in the S1 gene of infectious bronchitis virus. *Virus Research* **34** : 327 - 338

WANG, L., PARR, R. L., KING, D. J. AND COLLISSON, E. W. (1995) A highly conserved epitope on the spike protein of infectious bronchitis virus. *Archives of Virology* **140** : 2201 - 2213

WEBER, W. T. (1973) Direct evidence for the response of B and T cells to pokeweed mitogen. *Cellular Immunology* **9** : 482 - 487

WHITE, J. V. (1986) Cyclosporine: prototype of a T-cell selective immunosupressant. *Journal of the American Veterinary Medical Association* **189** : 566 - 569

WIGHT, P. A. L. AND SILLER, W. G. (1980) Pathology of deep pectoral myopathy of broilers. *Veterinary Pathology* **17** : 29 - 39

WILCOX, G. E., NANDAPALAN, N., FLOWER, R. L. P. AND FRYSMITH, D. (1983) Comparison of a microneutralisation test with ELISA and precipitin tests for detection of antibodies to infectious bronchitis virus in chickens. *Australian Veterinary Journal* **60** : 119 - 122

WILLIAMS, A., WANG, L., SNEED, L. W. AND COLLISSON, E. W. (1992) Comparative analyses of the nucleocapsid genes of several strains of infectious bronchitis virus and other corona viruses. *Virus Research* **25** : 213 - 222

WINTER, A.J. (1982) Microbial immunity in the reproductive tract. *Journal of the American Veterinary Medical Association* **181** : 1069 - 1073.

WINTERFIELD, R. W. AND ALBASSAM, M. A. (1984) Nephropathogenicity of infectious bronchitis virus. *Poultry Science* **63** : 2358 - 2363

WINTERFIELD, R. W. AND FADLY, A. M. (1972) Some characteristics of isolates of infectious bronchitis virus from commercial vaccines. *Avian Diseases* **16** : 746 - 755

WINTERFIELD, R. W., FADLY, A. M. AND BICKFORD, A. A. (1972) The immune response to infectious bronchitis virus determined by respiratory signs, virus infection and histopathological lesions. *Avian Diseases* **16** : 260 - 269

WINTERFIELD, R. W., FADLY, A. M. AND HOERR, F. J. (1976) Immunity to infectious bronchitis virus from spray vaccination with derivatives of Holland strain. *Avian Diseases* **20** : 42 - 48

WINTERFIELD, R. W. AND HITCHNER, S. B. (1962) Etiology of an infectious nephritis-nephrosis syndrome of chickens. *American Journal of Veterinary Research* **23** : 1273 - 1279

WITTER, R. L. (1962) The diagnosis of infectious bronchitis of chickens by agar gel precipitation test. *Avian Diseases* **6** : 478 - 492

WOLDEHIWET, Z. (1987) Depression of lymphocyte response to mitogens in sheep infected with tick-borne fever. *Journal of Comparative Pathology* **97**: 637 - 643

YACHIDA, S., AOYAMA, S., SAWAGUCHI, K., TAKAHASHI, N., IRITANI, Y. AND HAYASHI, Y. (1985) Relationship between several criteria of challenge-immunity and humoral immunity in chickens vaccinated with avian infectious bronchitis vaccines. *Avian Pathology* **14** : 199 - 211

YAGYU, K. AND OHTA, S. (1990) Detection of infectious bronchitis virus antigen from experimentally infected chickens by indirect immunofluorescent assay with monoclonal antibodies. *Avian Diseases* **34** : 246 - 252

YODER, H. W. JR., DRURY, L. N. AND HOPKINS, S. R. (1977) Influence of environment on airsacculitis: effects of relative humidity and air temperature on broilers infected with *Mycoplasma synoviae* and infectious bronchitis. *Avian Diseases* **21** : 195 - 208

ZANELLA, A (1988) Avian infectious bronchitis: properties and application of attenuated vaccine prepared with nephropathogenic strain AZ- 23/74. *Proceedings of the Ist International Symposium on Infectious Bronchitis,* Rauischholzhausen, Germany, pp. 335 - 342

ZELLEN, G. K. AND THORSEN, J. (1987) Determination of the antigenic relationship among six serotypes of infectious bronchitis virus using the enzyme linked immunosorbent assay and serum neutralisation test. *Avian Diseases* **31** : 455 - 458

ZHANG, S., LILLEHOJ, H. S. AND RUFF, M. D. (1995) *In vivo* role of tumour necrosis-like factor in *Eimeria tenella* infection. *Avian Diseases* **39** : 859 - 866

ZHANG, S. AND LILLEHOJ, H. S. (1995) Chicken tumour necrosis factor. I. *In-vitro* production by macrophages co-cultures with *Eimeria tenella* or stimulated with bacterial lipopolysaccharide. *Poultry Science* **74** : 1304 - 1310

ZIGTERMANN, G. J. W. J., VANDEVEN, W., VAN GEFFEN, C., LOEFFEN, A. H. C., PANHUIJZEN, J. H. M., RIJKE, E. O. AND VERMEULEN, A. N. (1993) Detection of mucosal immune responses in chickens after immunisation or infection. *Veterinary Immunology and Immunopathology* **36** : 281 - 291

ZWAAGSTRA, K. A., VAN DER ZEIJST, B. A. M. AND KUSTERS, J. G. (1992) Rapid detection and identification of avian infectious bronchitis virus. *Journal of Clinical Microbiology* **30** : 79 - 84

# APPENDIX

## BUFFERS, SOLUTIONS AND REAGENTS

### Antibiotic stock solution

| | |
|---|---|
| Crystapen<br>(Glaxo Lab. Ltd., Greenford, England) | 600 mg |
| Streptomycin sulphate BP<br>(Evans Medical Ltd., Greenford, England) | 1.0 g |
| Sterile distilled water | 40 ml |

4 ml of this stock solution was added to 1000 ml of medium for a 1x solution. For preparing 10x medium, 4 ml was added to 100 ml of medium.

### Phosphate buffered saline (PBS)

| | |
|---|---|
| Sodium chloride | 80 g |
| Potassium chloride | 2 g |
| Sodium hydrogen phosphate | 11.5 g |
| Potassium dihydrogen phosphate | 2 g |
| Distilled water | 800 ml |

## PREPARATION OF A CONTINUOUS SUCROSE GRADIENT

Initially, a 66% sucrose solution (855 g of sucrose dissolved in 495 mls of distilled water) was prepared using a magnetic stirrer. A range of sucrose solutions was then made up by diluting the 66% sucrose as described on the next page.

| % sucrose | Volume of 66% sucrose (ml) | Water (ml) |
|---|---|---|
| 55 | 40.00 | 7.75 |
| 50 | 36.00 | 11.75 |
| 45 | 32.00 | 15.75 |
| 40 | 27.75 | 20.00 |
| 35 | 24.00 | 23.75 |
| 30 | 20.00 | 27.75 |
| 25 | 16.25 | 31.50 |
| 20 | 12.50 | 35.25 |

To each sucrose solution the following buffer constituents were added:
1.25 ml of 4.0 M NaCl
0.5 ml of 1.0 M Tris-HCl pH 7.5
0.5 ml of 0.1 M EDTA pH 7.5

The whole range of sucrose solutions was used to make the gradient. Using a long-form pasteur pipette (tip positioned at the bottom of the centrifuge tube) and a syringe and needle, 3.5 mls of the 20% sucrose solution was added first. Then, similar volumes of 25, 30, 35, 40, 45, 50 and 55% sucrose solutions were carefully layered on the top and left overnight at $4^0$C, so that the solutions diffused and formed a continous gradient. This gradient was used for the purification of IBV.

## ELISA REAGENTS

### Coating buffer

| | |
|---|---|
| $NaCO_3$ | 1.5 g |
| $Na_2HCO_3$ | 2.93 g |

Made up to 1 litre and stored for up to a week at $4^0$C

**Washing buffer**

| | |
|---|---|
| NaCl | 292.2 g |
| $Na_2HPO_4$ (anhydrous) | 5.35 g |
| $Na_2HPO_4$. 12 $H_2O$ | 13.4 g |
| or $NaH_2PO_4$. 2 $H_2O$ | 1.95 g |
| Tween 20 | 5.0 ml |

Made up to to 5 litres.
This was diluted to half the concentration for use.

**Phosphate-citrate buffer**

| | |
|---|---|
| 0.1M citric acid | 243 ml |

0.2M $Na_2HPO_4$ added till pH 5.0
Make up to 1 litre with distilled water.

**OPD substrate**

0.4 g of OPD dissolved in phosphate-citrate buffer was divided into 12 ml aliquots and stored at -20$^0$C. Prior to use, 6 ml of 30% hydrogen peroxide was added to 12 ml of thawed substrate and used.

**Stop solution**

2.5N sulphuric acid was used.

## IMMUNOFLUORESENCE STAINING

**Dilution buffer**

PBS with 1% bovine serum albumin (fraction V) was used.

## IMMUNOPEROXIDASE STAINING

Removal of endogenous peroxidase was done by immersing slides in 150 ml of PBS with 1 ml of freshly-prepared 6% hydrogen peroxide for 10 minutes.

**Blocking solution for Fc receptors**

For virus antigen detection, PBS with 0.05% tween 20, 0.1% bovine serum albumin, 0.001% sodium azide and 20% normal chicken serum was used as a blocking buffer.

**Substrate solution**

10 mg tablets of diaminobenzidine (Dako Ltd., Bucks, UK) were dissolved in 10 ml of PBS, filtered and used fresh. To every 2 ml of this solution 15 µl of 3% $H_2O_2$ was added before use.

**TOC MEDIUM**

100 ml of 10x MEM (Life Technologies, Paisley, Scotland)
20 ml of 7.5% sodium bicarbonate (Life Technologies)
900 ml of sterile distilled water
4 ml of stock antibiotic solution

**OOC AND CEF MEDIUM**

85 ml of 10x M199 (Life Technologies)
100 ml of tryptose phosphate broth (Sigma)
10 ml of 7.5% sodium bicarbonate (Life Technologies)
14 ml of HEPES (Life Technologies)
625 ml of sterile distilled water
4 ml of stock antibiotic solution

**TRYPSIN SOLUTION FOR CEF PRIMARY CULTURE**

| | |
|---|---|
| Trypsin (1:250) solution (Life Technologies) | 10 ml |
| PBS | 90 ml |

Trypsin solution was aliquoted in 10 ml amounts and stored at -20$^0$C. Immediately before use, one aliquot was thawed and diluted in 90 ml of pre-warmed PBS.

**NATT AND HERRICK'S SOLUTION** (for total white blood cell count)

| | |
|---|---|
| NaCl | 3.88 g |
| $Na_2SO_4$ | 2.50 g |
| $Na_2HPO_4$. 12 $H_2O$ | 2.91 g |
| $KH_2PO_4$ | 0.25 g |
| Formalin (37%) | 7.50 ml |
| Methyl violet 2B | 0.1 g |

Make up to 1 litre. Allow the solution to stand overnight and filter before use.