PATHOGENESIS OF AVIAN REOVIRUS INFECTION

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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January, 1988

To

My wife

PREFACE

The experimental work described in this thesis was conducted in the Department of Veterinary Pathology, University of Liverpool, between 1985 and 1987, during the tenure of a scholarship awarded to the author by the Commonwealth Scholarship Commission.

ACKNOWLEDGMENTS

I am grateful to my supervisors, Dr. R.C. Jones and Professor D.F. Kelly for their guidance, advice, encouragement and willing help throughout the experimental work and in the preparation of the thesis.

I should also thank Dr. F.T.W. Jordan, Dr. J.M. Bradbury, Dr. Z. Woldehiwet, Dr. S.D. Carter and other members of the Department with whom I had many useful discussions.

I acknowledge with thanks and appreciations the excellent technical assistance of Mrs. C.E. Savage and Mrs. S.M. Spiller. I also thank Mr.A. Teasdale, Mrs. F. Copley and Mr. A. Jopson for their assistance in the management of experimental chickens. My thanks are also extended to other members of the technical staff of this laboratory for their assistance and co-operation.

I would like to thank Mr. G. Ross for the preparation of tissue sections for electron microscopy and Mr. B. Getty and Dr. R.A. Williams for electron photomicrography. I should also thank Mrs. P. Jenkins for the preparation of beautiful photomicrographs of histological sections.

I wish to take this opportunity to express my sincere appreciation to all fellow post-graduate students of Leahurst for their help and memorable company.

Finally, I am grateful to the Commonwealth Scholarship Commission for the financial support.

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Abstract

Avian reoviruses have been isolated from chickens with a variety of disease conditions. The most important of these is tenosynovitis (viral arthritis) where a relationship between the virus and disease has been established. The present thesis investigated some aspects of the pathogenesis of experimental reovirus infection in chickens.

The first part of the work describes the standardization of immunohistochemical techniques including immunofluorescence and immunoperoxidase staining for tracing virus in tissues as a basis for later pathogenesis studies. In addition, an indirect enzyme-linked immunosorbent assay was developed to measure reovirus antibody titres in a single dilution of serum.

Early events in reovirus pathogenesis were studied by virological, immunohistochemical and electron microscopic methods. These showed viral entry and primary replication in the epithelium of the intestine and bursa of Fabricius within 12 hours following oral infection. These were followed by a rapid pantropic distribution of virus which reached most tissues within 24 to 48 hours after infection.

Following infection of day-old chicks by different routes, cloacal virus shedding continued for about 3 to 4 weeks and virus persisted in hock (tibiotarsal-tarsometatarsal) joints until the end of the experiment (5 weeks). Although the isolation of virus from the joint was often difficult in the later stages of infection, joint lesions still continued to progress. When compared with oral or subcutaneous infection, direct inoculation of virus into the hock joint or foot-pad enhanced the disease process with the development of cartilage lesions as early as 2 to 3 weeks after infection.

Young chicks infected at 1-day of age did not seroconvert until 2 to 3 weeks later, but in mature chickens antibodies were detected as early as 1 week post-infection. Chickens infected at 1-day of age developed a delayed-type hypersensitivity reaction at 6 weeks of age following challenge with viral antigen. Maternal antibodies appeared to protect newly-hatched chicks against reovirus infection by preventing viral spread to target tissues, although primary replication in the intestine was unaffected.

Preliminary studies provided evidence for the development of autoantibodies, such as antinuclear and anti-collagen antibodies following reovirus infection, but rheumatoid factor was not detected. Immune complexes were present in the sera of infected chickens.

The evidence from this study supported the proposal that reovirus-induced arthritis of chickens could be a valuable model for rheumatoid and other forms of chronic arthritis with particular reference to investigation of the pathogenesis of synovial injury associated with infection or allergy.

ABBREVIATIONS USED

ABC	avidin-biotinylated enzyme complex
AC	allantoic cavity
ACA	anti-collagen antibody
AEC	aminoethylcarbazole
AGP	agar gel precipitation
ANA	antinuclear antibody
CAM	chorio-allantoic membrane
CEF	chicken embryo fibroblast
CELi	chicken embryo liver
CELu	chicken embryo lung
CK	chicken kidney
CPE	cytopathic effect
CV	coefficient of variation
DAB	diaminobenzidine
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
FA	fluorescent antibody
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
HE	hematoxylin and eosin
HVT	herpes virus of turkey
IP	immunoperoxidase
LTA	lymphocyte transformation assay
OPD	o-phenylenediamine
PAP	peroxidase-antiperoxidase

PBS	phosphate buffered saline
PEG	polyethylene glycol
PHA	phytohaemagglutinin
p.i.	post infection
PNT	positive-negative threshold
PVC	polyvinyl chloride
RF	rheumatoid factor
SPF	specific pathogen free
TBS	tris buffered saline
$TCID_{50}$	50% tissue culture infective dose
TNE	tris-NaCl-EDTA
UV	ultraviolet
VBS	veronal buffered saline
VN	virus neutralization
v/v	volume/volume
w/v	weight/volume
YS	yolk sac

Chapter I

INTRODUCTION

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Chapter I

INTRODUCTION

In 1954, an infectious agent, different from mycoplasmas and presumed to be a virus, was isolated from outbreaks of synovitis in chickens (Olson et al., 1954). The agent was shown to be capable of reproducing synovitis and arthritis in chickens following experimental infection (Olson et al.,1956,1957).This viral arthritis agent was finally identified as an avian reovirus (Walker et al., 1972). Following the original isolation and characterization of the virus, outbreaks of viral arthritis or tenosynovitis in chickens have been reported from many parts of the world (Kibenge and Wilcox, 1983; Robertson and Wilcox, 1986). In the UK, an avian arthrotropic reovirus was first isolated from chickens with tenosynovitis and ruptured gastrocnemius tendon (Jones et al., 1975).

Apart from isolation and characterization of the virus, efforts have also been made to understand the nature of the disease. The relevant literature on the epidemiology and pathogenesis of reovirus-induced tenosynovitis (viral arthritis) in chickens is reviewed here following a brief account of the structure and properties of the virus.

1. STRUCTURE AND PROPERTIES OF AVIAN REOVIRUSES

1.1. Family Reoviridae

The name 'reovirus' (respiratory enteric orphan viruses) was proposed by Sabin (1959) for a group of viruses that were typically isolated from the respiratory and enteric tracts of children and were not associated with any known disease condition. Later, several other groups of viruses with common structural features were included in the reovirus family. At present the family Reoviridae consists of six genera: reovirus (orthoreovirus), orbivirus, rotavirus, cypovirus, phytovirus and fijivirus (Joklik, 1983). The first three genera include viruses which infect man and animals, and the remaining three genera contain plant and insect viruses.

General characteristics of the family Reoviridae have been summarized by Tyler and Fields (1986a). The viruses belonging to the family form nearly spherical icosahedrons of 70 nm in diameter; they are non-enveloped and possess a double protein capsid. The genome consists of 10-12 segments containing double-stranded RNA. The replication cycle of the family Reoviridae is characterized by fully cytoplasmic multiplication, inclusion body formation, lack of complete uncoating of virions, full length capped (5'), nonpolyadenylated mRNA transcript, and

possession of all enzymes required for dsRNA transcription (Tyler and Fields, 1986a).

1.2. Avian reoviruses

The genus reovirus can be divided into two main groups in which there are mammalian and avian members. The avian reoviruses, unlike their mammalian counterparts, produce syncytia in cell culture and lack haemagglutinating activity (Kawamura et al., 1965; Deshmukh and Pomeroy, 1969a). The Nelson Bay virus, isolated initially from a flying fox Petropus poliocephalus (Gard and Compans, 1970), is of mammalian origin and shares the group-specific antigen of mammalian reoviruses but has cell fusion activity like avian reoviruses (Gard and Marshall, 1973).

The Fahey-Crawley agent, a virus isolated from the respiratory tract of a chicken with chronic respiratory disease (Fahey and Crawley, 1954), was characterized by Petek et al. (1967) as the first avian reovirus isolate. Kawamura et al. (1965) isolated and characterized 77 avian reovirus isolates from chickens with or without respiratory and enteric disease conditions. Although avian arthrotropic reoviruses were originally isolated in the 1950s, they remained unidentified until 1972 (Walker et al., 1972).

1.2.1. Structure and physico-chemical properties of avian reoviruses

The morphology and physico-chemical properties of avian reoviruses have been studied by many investigators (Kawamura et al., 1965; Petek et al., 1967; Deshmukh and Pomeroy, 1969b; Koide, 1970; Mustaffa-Babjee et al., 1973; Meulemans and Halen, 1982). The genome and protein structure of avian reoviruses have been studied by Spandidos and Graham (1976) and Schnitzer et al. (1982). Haemagglution by avian reoviruses was attempted without success by Taylor et al. (1966); Deshmukh and Pomeroy (1969a) and Mustaffa-Babjee et al. (1973). The main features of the structure and physico-chemical properties of avian reoviruses are summarized in Table I.1.

1.2.2. Serotypes of avian reoviruses

While mammalian reoviruses have been classified into three distinct serotypes on the basis of neutralization and haemagglutination tests (Rosen, 1960), the serotyping of avian reoviruses appears to be very difficult because of marked heterogenicity in their neutralizing antigens. Furthermore, avian reoviruses lack haemagglutination properties. Kawamura et al. (1965) classified 77 Japanese avian reovirus isolates into five serotypes. Sahu and Olson (1975) and Sahu et al. (1979) grouped 10 American isolates

Table I.1. Structure and physico-chemical properties of avian reoviruses

Structure
58-82 nm in diameter
Non enveloped
Hexagonal icosahedron
Double protein capsid shell
Segmented dsRNA genome (10 segments)
Physico-chemical properties
Density: 1.36-1.37g/ml (virion)
1.29-1.30g/ml (top component)
1.44g/ml (core)
Stable at pH 3-9 for 1-4 hours
Stable at 50°C for 2 hours
Resistant to ether, chloroform, trypsin and sodium deoxycholate
Sensitive to phenol, mercury bichloride, ethanol, tincture of iodine and chlorine
Partially resistant to formalin at low temperature
Do not haemagglutinate

into four serotypes. Wood et al.(1980) grouped six avian reovirus isolates from Britain, Germany and USA into three serotypes, and compared them with five Japanese and four American prototype strains. They claimed the existance of at least 11 avian reovirus serotypes. However, Green et al.(1976) found that five New Zealand isolates of avian reoviruses belonged to a single serological group. Robertson and Wilcox (1984) considered 10 avian reovirus isolates of Australian origin to fall into a single serotype, but divided them into three subtypes.

1.2.3. Cultural characteristics of avian reoviruses

The Fahey-Crawley agent (Fahey and Crawley, 1954) and viral arthritis agent (Olson et al., 1954), subsequently both identified as avian reoviruses, were initially isolated in chicken embryos by yolk sac (YS) inoculation, although Fahey and Crawley (1954) also used the allantoic cavity (AC) route. Taylor et al. (1966) adapted the viral arthritis agent to grow in chicken embryo fibroblast (CEF) cell culture. Petek et al.(1967) used chicken embryo lung (CELu) cell culture to grow and characterize the Fahey-Crawley agent. Kawamura et al.(1965) isolated avian reoviruses in chicken kidney (CK) cell culture. Deshmukh and Pomeroy (1969a) isolated avian reoviruses in avian embryos by the chorioallantoic membrane (CAM) route of infection.

Jones et al.(1981) used chicken embryo liver (CELi) cell culture for isolation of avian reoviruses. Guneratne et al.(1982) compared various systems including CELi, CELu, CEF and CK cell cultures and also chicken embryos inoculated through YS, CAM and AC routes for primary isolation and cultivation of avian reoviruses. They found CELi cell culture and the YS route of embryo inoculation to be the most sensitive systems for isolation and cultivation of avian reoviruses.

Avian reoviruses have been adapted to grow in several established mammalian cell lines such as African green monkey kidney (VERO), baby hamster kidney (BHK), Georgia bovine kidney (GBK), Crandall feline kidney (CRFK), rabbit kidney (RK) and porcine kidney (PK) cell lines (Barta et al.,1984).

Growth of avian reoviruses in chicken embryos is indicated by embryo mortality within 3-5 days with extensive subcutaneous haemorrhage or thickened and oedematous CAM containing necrotic plaques following inoculation by YS or CAM route, respectively. Surviving embryos become stunted with hepatitis, pericarditis and splenomegaly (Mustaffa-Babjee et al., 1973; Guneratne et al.,1982).

In cell culture avian reoviruses produce syncytia and eosinophilic intracytoplasmic inclusion bodies of different shapes and sizes (Kawamura et al.,1965; Petek et al.,1967).

2. EPIDEMIOLOGY OF AVIAN REOVIRUS INFECTION

2.1. Avian reoviruses and disease

Avian reoviruses have been isolated from chickens with a variety of disease conditions including respiratory disease (Fahey and Crawley,1954), arthritis or tenosynovitis (Olson et al.,1954), enteric disease (Kawamura et al.,1965), hydropericardium (Spradbrow and Bains,1974) runting or malabsorption syndrome (Kouwenhoven et al.,1978), and also from clinically normal chickens (Robertson et al.,1984). Following these initial reports, isolation of avian reoviruses from chickens has been documented in many parts of the world (Kibenge and Wilcox, 1983; Robertson and Wilcox,1986). In the UK, isolations of reovirus have been made from chickens with tenosynovitis (Jones et al.,1975; Macdonald et al.,1978; Jones et al., 1981), hydropericardium (Jones, 1976), respiratory disease (McFerran et al.,1971) and stunting syndrome (Bracewell and Wyeth,1981).

Avian reoviruses also have been isolated from turkeys with enteric disease (Fujisaki et al.,1969), turkey-poult mortality (Simmons et al.,1972) and tenosynovitis (Page et al.,1982), from Muscovy ducks with diarrhoea, stunting and mortality (Kaschula, 1950), from apparently healthy Mallard ducks (McFerran et al.,1976), from pigeons with hepatitis (Vindevogel et al.,1982) and diarrhoea (McFerran et al., 1976),

from geese with respiratory disease (Csontos and Miklovich-Kis Csatari,1967); and from psittacine birds with hepatitis, splenomegaly and pneumonia (Meulemans et al.,1983).

Avian reoviruses have been isolated from chickens with a variety of disease conditions. However, with the exception of viral arthritis or tenosynovitis, the aetiological relationship between the virus isolation and these diseases has not been fully established. Viral arthritis or tenosynovitis is primarily a disease of broiler chickens affecting mainly the hock (tibiotarsal-tarsometatarsal) joints, gastrocnemius and digital flexor tendons and tendon sheaths. The disease causes swelling at or around the hock joints, lameness and occasional rupture of the gastrocnemious tendon (Kibenge and Wilcox, 1983).

At this point it is worth briefly mentioning that the role of mammalian reoviruses in disease is also difficult to ascertain, although 50 to 80 per cent of the adult human population has antibodies to reovirus (Stanley,1977). Mammalian reoviruses have been suspected to be associated with mild respiratory and gastrointestinal infection (Rosen et al.,1963) and neonatal biliary atresia in humans (Bangaru et al.,1980), but there is little evidence for a causal relationship between these diseases and reovirus. In mice, however, reovirus causes a

spontaneous disease characterized by diarrhoea, oily hair, growth retardation, jaundice and ataxia (Sharpe and Fields,1985). In addition, various syndromes can be induced experimentally in suckling mice with reoviruses (Tyler and Fields, 1986a).

2.2. Transmission of avian reoviruses

It has been shown experimentally that avian reoviruses can spread from infected to in-contact chickens (Kerr and Olson,1969; Olson and Khan,1972; van der Heide et al.,1974; Sahu and Olson,1975; Jones and Onunkwo,1978; Macdonald et al.,1978). Apart from contact exposure, many other experimental routes have been used for reovirus infection in chickens. These include footpad, intramuscular, intravenous (Olson, 1959), intratracheal (Olson and Solomon,1968), intranasal (Olson and Weiss,1972), intraperitoneal (Johnson,1972), oral, and subcutaneous (Glass et al., 1973) routes of infection. As avian reoviruses are ubiquitous, relatively resistant to heat and disinfectants and readily shed in the faeces of infected birds, food and drinking water in commercial poultry housings may easily become contaminated and serve as a principal source of infection.

Avian reoviruses can also be transmitted vertically. The evidence for the ability of avian reoviruses to survive in embryos came from the findings that the virus can be isolated from chicks

infected as embryos (Menendez et al.,1975a; Hussain and Spradbrow,. 1981; Jones and El-Taher,1985), and also from the report that the virus was detected in cell cultures prepared from eggs laid by experimentally infected chickens (van der Heide and Kalbac, 1975). However, the direct evidence for egg-transmission of avian reoviruses was provided by Menendez et al, (1975a), who were able to reisolate the virus from three chicks hatched from eggs laid by an experimentally infected hen.

2.3. Effects of age and breed of chickens on reovirus infection

An age-related resistance of chickens to avian reovirous infection was first observed by Kerr and Olson(1964). They demonstrated that 15 and 20 weeks old chickens showed marked resistance to infection as compared to 2, 5 and 10 weeks old chickens. Wood and Thornton (1981) reported that day-old chicks were more susceptible to reovirus infection, by both foot-pad and oral routes, when compared with 2 weeks old chickens. Age-related resistence of chickens to reovirus infection was conclusively demonstrated by Jones and Georgiou (1984). They infected groups of chickens by oral and foot-pad routes at 1-day, and 2, 4, 6 and 9 weeks of age, and observed a graded response. The severity of the disease in terms of clinical signs, gross and microscopic lesions and

viral persistence decreased with the increase in age at infection. However, the basis for the age-related resistance of chickens to reovirus infection is not known, but Jones and Georgiou (1984) suggested that it could be related to the developing immuno-competence of growing chickens.

Reoviral tenosynovitis is primarily a disease of meat-type chickens, although the disease has been observed occasionally in light-breed chickens (Schwartz et al.,1976; Macdonald et al.,1978), and the disease can be reproduced experimentally in light breed chickens (Jones and Onunkwo, 1978). The relative susceptibility of different breeds of chickens to avian arthrotropic reovirus infection has been studied by Jones and Kibenge (1984). Although most infected birds of both light and broiler breeds developed the disease to some extent, the more severe clinical disease simulating natural cases of tenosynovitis with ruptured gastrocnemius tendon was observed only in broiler chickens. The basis for the greater susceptibility of broiler chickens to reovirus-induced tenosynovitis is not known. It has been suggested that greater body weight of broiler chickens might predispose to the disease and rupture of load-bearing leg tendons (Kibenge and Wilcox, 1983). The fibrous connective tissues of tendons and joints of broiler chickens are reported to be inferior to those of layer breed chickens in terms of tensile strength

(van Walsum,1975), histological compactness (van Walsum,1977) and the degree of interaction between collagen and glycosaminoglycans (van Walsum et al., 1981). These structural and functional properties of leg tendons were considered to be responsible for the greater susceptibility of broiler chickens to rupture following synovitis.

3.PATHOGENESIS OF REOVIRUS-INDUCED TENOSYNOVITIS

3.1. Early events in the pathogenesis

Although the early pathogenesis of mammalian reovirus infection has been studied extensively (Sharpe and Fields,1985), very little is known about the portal of entry, site of primary replication and mode of spread of avian reoviruses following infection in chickens. Menendez et al.(1975b) observed a wide-spread distribution of reovirus in the respiratory, alimentary, reproductive tissues and tendons of hock joints of mature chickens at 4 days after infection via respiratory and oral routes. Ellis et al.(1983) infected 6-days old and 4-weeks old chickens with an avirulent strain of reovirus and the Fahey-Crawley virus and reisolated the virus from the lung, liver, heart, a pool of lymphoid tissues and serum at different times during the first 5 days after infection by various routes. A wide distribution of reovirus in enteric, parenchymatous

and joint tissues of chickens during the early stage of oral infection of day-old chickens was reported by Kibenge et al.(1985). They demonstrated a viraemia, which appeared as early as 24 hours after infection; this was mostly associated with plasma, although virus was detected occasionally in the blood mononuclear fraction. Similar pantropic distribution of virus in various tissues has been observed in suckling mice following infection with mammalian reovirus (Kundin et al.,1966).

3.2. Persistence of avian reoviruses in tissue

Prolonged persistence of avian reovirus in tissues was first reported by Olson and Kerr (1967) who infected chickens at two weeks of age with reovirus and were able to isolate the virus from the hock joints of infected birds 285 days after infection. The evidence for virus persistence was further supported by Jones and Onunkwo (1978) and Marquardt et al. (1983), who were able to reisolate the virus from the hock joints of chickens, that had been infected as day-olds, upto 13 weeks and 115 days, respectively. The virus was also found to persist in the foot-pad upto 120 days (Olson and Kerr,1967) and 7 weeks (Jones and Onunkwo,1978) after infection by injection of virus at that site. The persistence of the virus in other tissues is not known, although there is a report of occasional reisolation from

the liver and spleen upto 34 and 235 days after infection, respectively (Olson and Kerr,1967). The cloacal shedding of virus usually ceases 2 to 3 weeks after infection, but could continue intermittently upto 7 weeks after infection (Mukiibi-Muka et al.,1984; Jones and Georgiou,1984; Jones and Kibenge,1984).

Mammalian reoviruses can produce persistent infection in a variety of cell cultures (Bell and Ross,1966; Ahmed and Graham,1977). This phenomenon has been suggested to be associated with mutation of a genome segment of the virus (Ahmed and Fields, 1982). Persistent infection of cell culture with avian reoviruses has not yet been recorded.

3.3. Pathology of avian reovirus infection

3.3.1. Natural tenosynovitis-arthritis

Natural cases of viral tenosynovitis of chickens have been characterized by enlargement and induration of tendons above or below the hock joints and occasional rupture, with haemorrhage, of the gastrocnemius tendon (Olson and Solomon,1968; Johnson and van der Heide,1971: Jones et al.,1975; Itakura et al.,1977).

3.3.2. Experimental tenosynovitis-arthritis

The pathology of reovirus-induced experimental tenosynovitis-arthritis has been studied after infection by the foot-pad route in 2 or 3 weeks old chickens (Kerr and Olson,1969; Macdonald et al.,1978)

and also in day-old chicks (Jones and Onunkwo, 1978; Jones and Kibenge, 1984). Other modes of infection, such as, intramuscular, intraperitoneal (Johnson, 1972), oral and subcutaneous routes (van der Heide et al., 1974) and contact exposure (Kerr and Olson, 1969; Jones and Onunkwo, 1978; Macdonald et al., 1978) have been used for the study of experimental tenosynovitis in chickens. Although the lesions described by all investigators were broadly similar, they varied in severity and course of development, probably due to variations in the age and breed of chickens, routes of infection and the dose and virulence of the virus strains used.

The initial gross lesions of experimental tenosynovitis appear within 4 days to 4 weeks as an oedematous swelling in the areas of the digital flexor tendons and metatarsal extensor tendons (Kerr and Olson, 1969; Jones and Onunkwo, 1978). The inflammatory exudates between tendons have been described as purulent (Kerr and Olson, 1969), serous (Jones and Onunkwo 1978) or yellowish brown and gelatinous (Jones and Kibenge, 1984). Petechial haemorrhages along tarsal and metatarsal tendons and in the synovial membrane of hock joints have been observed by Kerr and Olson (1969) 9 days after foot-pad infection. The hock joint usually contains small amount of straw-coloured or blood-tinted exudate (Olson, 1978).

The inflammation in tendon areas may be followed by the rupture of digital flexor (Jones et al., 1980) and gastrocnemious tendons (Jones and Kibenge, 1984), but more frequently progresses to chronic tenosynovitis characterized by thickening, hardening and fusion of tendons and tendon sheaths (Kerr and Olson, 1969; Jones and Onunkwo, 1978; Jones and Kibenge, 1984). Small pitted erosions can develop in the articular cartilage of the distal tibiotarsus about 6 weeks after foot-pad infection (Kerr and Olson, 1969; Jones and Guneratne, 1984) or 12 weeks after oral infection (Jones and Kibenge, 1984).

Microscopic lesions of reovirus-induced tenosynovitis are initially those of granulomatous inflammation but later there is repair and sometimes degeneration of the tendon. During the first few weeks after infection the histopathological changes in the tendon sheaths and synovium include oedema, infiltration of mononuclear cells and heterophils, hypertrophy and hyperplasia of synovial cells, formation of lymphoid nodules and synovial villi (Kerr and Olson, 1969; Jones and Onunkwo, 1978; Macdonald et al., 1978; Jones and Guneratne, 1984). Later in the course of the disease, the amount of fibrous connective tissue gradually increases in tendons and tendon sheaths (Kerr and Olson, 1969; van der Heide et al.,1974; Jones and Onunkwo, 1978). Other reported osseous changes in the hock joints of reovirus- infected

chickens include narrow and irregular cartilage growth zones, erosions of cartilage followed by fibro-cartilaginous pannus formation and increased osteo-blastic activity, and inhibition of sesamoid bone development (Kerr and Olson, 1969).

3.3.3. Experimental hepatitis and myocarditis

Although Kerr and Olson (1969) did not observe any hepatic lesions in chickens infected with reovirus at 2 weeks of age, Mandelli et al. (1978) were able to induce experimental reovirus-hepatitis in newborn chicks. Jones and Guneratne (1984) recorded deaths associated with hepatic necrosis in young chicks during the first few days of life following experi-mental infection at day-old with high doses of various strains of avian reoviruses. The description of reovirus-hepatitis has been given by Mandelli et al. (1978); grossly, the liver was moderately enlarged, discoloured and haemorrhagic, and contained scattered white or yellow necrotic foci. Microscopically, hydropic degeneration, focal necrosis, haemorrhage and polykaryocytes were observed. The necrotic areas were later invaded by a heterogeneous cell population most of which were macrophages and regenerating hepatocytes.

Experimental reovirus-induced myocarditis has been reported by Kerr and Olson (1967). Microscopic lesions of myocarditis included oedema, infiltration

of heterophils and mononuclear cells, proliferation of reticuloendothelial cells and degeneration of muscle fibres.

3.3.4. Pathology of avian reovirus infection in mice

Experimental infection of newborn mice by the oronasal route with an avian reovirus (Fahey-Crawley virus) produced hepatitis, congestive and haemorrhagic encephalomyelitis and severe suppurative broncho-pneumonia apparently caused by secondary bacterial invasion with endogenously derived Pasteurella pneumotropica (Phillips et al., 1970).

3.4. Immune responses of chickens to reovirus infection

Various serological tests have been used to detect and quantitate humoral immune response of chickens to avian reoviruses. These include agar gel double diffusion or agar gel precipitation (AGP) test (Olson and Weiss, 1972), virus-neutralization on the basis of reduction of plaque formation in cell cultures (Kawamura et al., 1965) or on the chorio-allantoic membrane of chicken embryos (Deshmukh and Pomeroy, 1969a), virus microneutralization on the basis of reduction in cytopathic effects (CPE) in cell cultures (Rau et al., 1980; Jones and Kibenge, 1984) and an enzyme-linked immunosorbent assay (ELISA) (Slaght et al., 1978).

Following experimental inoculation precipitating and neutralizing antibodies usually appear in serum 2 to 3 weeks after infection (van der Heide et al., 1974; Jones and Onunkwo, 1978; Mukiibi-Muka et al., 1984). However, the seroconversion may be enhanced or delayed depending on the age of birds at infection, breed of chickens and route of infection used. The positive AGP reaction may be observed as early as one week after infection if the chicks are infected at 2 weeks of age or older (Jones and Georgiou, 1984). The proportion of birds showing positive AGP reaction is usually higher after infection by parenteral routes than after oral infection (van der Heide et al., 1974; Jones and Georgiou, 1984). Jones and Kibenge (1984) reported that virus-neutralizing antibodies appeared in the lighter breeds at 3 weeks but in broilers at 4 weeks after infection. Chicks receive maternal antibodies from hens exposed to the virus (van der Heide et al., 1976).

Very little is known about the cell-mediated immune response of chickens to reovirus infection. Kibenge et al. (1987) studied the effects of surgical and chemical immunosuppression on the reovirus-induced tenosynovitis in chickens and suggested that recovery from reovirus infection probably involved both B- and T- cell systems but B-cell system was predominantly protective. In vitro production of interferon in CEF cell culture and activation of cultured

macrophages with avian reovirus have been reported by von Bülow et al. (1984).

It has been proposed that reovirus-induced arthritis in chickens could be useful as a model for human rheumatoid arthritis (Walker et al., 1977; Marquardt et al., 1983). However, the attempts to demonstrate rheumatoid factor in reovirus-induced arthritis have been unsuccessful (Taylor, 1965; Walker et al., 1977).

Recently Pradhan et al. (1987) reported the presence of antinuclear antibody in the serum of chickens infected with reovirus.

Newborn chicks hatched from flocks exposed to reovirus obtain maternal antibodies from their yolk sacs. This phenomenon has been used for vaccinating breeder chickens to protect their progeny (Cessi and Lombardini, 1975). Various types of vaccines, including inactivated (Cessi and Lombardini, 1975; Wood and Thornton, 1981), live virulent (van der Heide, 1975) and partially attenuated (van der Heide et al., 1976) reoviruses have been used to vaccinate breeder chickens. These vaccination trials often suffer from the problems associated with weak protection (van der Heide et al., 1976; Wood and Thornton, 1981), lack of cross protection to certain strains (Rau et al., 1980) and danger of vertical transmission (van der Heide et al., 1983). The development of a fully attenuated vaccine and a temperature-sensitive

mutant vaccine were reported by van der Heide et al. (1983) and Haffer (1984), respectively. Both vaccines were non-pathogenic for 1-day old chicks.

There are conflicting reports on the ability of avian reoviruses to cause immunosuppression in chickens. van der Heide et al. (1983) observed an increased incidence of Marek's disease after simultaneous vaccinations of chickens at 1-day of age against Marek's disease with herpesvirus of turkeys (HVT) and with avian reovirus. This observation was further supported by Rinehart and Rosenberger (1983), who observed a four times higher condemnation rate due to Marek's disease among chickens vaccinated simultaneously with HVT and reovirus than in chickens vaccinated with HVT alone. However, the suggestion that avian reovirus interferes with the development of immunity to Marek's disease was not supported by Eidson and Kleven (1983), who observed protection against challenge with both reovirus and Marek's disease virus following simultaneous vaccination with HVT and reovirus. Cho (1979) studied the effects of avian reovirus on Marek's disease and found that preexposure of chickens to avian reovirus reduced the incidence of gross lesions and/or mortality due to experimental Marek's disease.

Rinehart and Rosenberger (1983) studied the effects of 5 strains of avian reoviruses, including non-pathogenic, moderate and highly virulent strains,

on the immune functions of chickens. They found that avian reoviruses did not interfere with the response of chickens to Brucella abortus, a T-independent antigen, but the response to sheep red blood cells, a T-dependent antigen either decreased or elevated depending on the strains of reovirus used. Cook and Springer (1983) reported that avian reovirus did not adversely affect the immunocompetence of 8 to 12 weeks old chickens as evaluated by haemagglutinating response to sheep red blood cells and delayed hypersensitivity response to phytohaemagglutinin. Montgomery et al. (1986) reported that subcutaneous inoculation of avian reovirus in newly hatched chicks resulted in altered organ weight, such as an increase in the weight of the spleen but a decrease in the weight of the bursa of Fabricius. In addition there was an increase in total leucocyte count, a reduced mitogen response of peripheral blood leucocytes to phytohaemagglutinin and reduced phagocytosis by monocytes. The serological responses to Brucella abortus, Newcastle disease virus or sheep red blood cells were not significantly affected. Montgomery et al. (1986), however, considered these findings to be insufficient to support the hypothesis that avian reovirus could cause general immunosuppression.

4. AIM OF THE THESIS

It would appear from the literature reviewed above that avian reoviruses can cause tenosynovitis and arthritis in chickens and these are characterized by chronic localized granulomatous lesions in the hock joints. Very little is known, however, about the pathogenesis of reovirus-induced chronic tenosynovitis and arthritis which ultimately may lead to the rupture of tendons. The aim of this thesis, therefore, is to investigate some aspects of the pathogenesis of avian reovirus infection which might contribute to better understanding of reovirus-induced tenosynovitis. Such information could also shed light on the mechanisms of chronic arthritis of unknown cause in other species.

The first part of the thesis describes the development, adaptation and standardization of some immunological and immunohistochemical techniques which are utilized in the subsequent studies. The rest of the thesis describes studies on the pathogenesis of experimental reovirus infection in chickens. The major objectives of these studies are as follows:

a) to investigate early events in the pathogenesis of avian reovirus infection

b) to examine the pathology of reovirus-induced tenosynovitis with special reference to the influence of the route of experimental infection

c) to study the immune responses of chickens in reovirus infection and their possible involvement in the pathogenesis of reovirus-induced tenosynovitis and arthritis.

Chapter II

GENERAL MATERIALS AND METHODS

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Chapter II

GENERAL MATERIALS AND METHODS

This chapter describes the general materials and methods used throughout the whole investigation. Details of more specialized techniques, where used, are given in relevant chapters. The formulae of commonly used reagents, media and buffers are given in Appendix-A.

1.PREPARATION OF GLASSWARE

All glassware was prepared according to routine protocol of the laboratory. This involved disinfection of used glassware which had been contaminated with infectious materials, followed by cleaning and sterilization.

1.1.Disinfection of used glassware

All glassware which had come in contact with any infectious materials was disinfected before cleaning and sterilization. Used graduated and pasteur pipettes were soaked overnight in a disinfectant containing sodium hypochlorite (Chloros)*. Pasteur pipettes were discarded after single use. All other contaminated glassware was collected in a stainless steel container and disinfected by autoclaving at 121°C for 15 minutes at a pressure of 15 lbs/sq. in. All used disposable

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* The Golden Grain Products Ltd., Liverpool, England.

plasticware was discarded and incinerated after disinfection by overnight soaking in an iodophor based disinfectant (FAM)*.

1.2. Cleaning of glassware

All used and new glassware was cleaned either manually by brushing, scrubbing and rinsing or automatically, using a glass washing machine (Gallay Lab 901)**, as appropriate. For manual cleaning, glassware was soaked in a liquid detergent (Micro)[a] for a few hours or overnight, then cleaned throughly by brushing and scrubbing, if needed and rinsed serially in changes of tap water and distilled and deionized water. The clean glassware was drained and dried in a hot air oven. Where convenient, glassware was washed in the washing machine, where washing with a detergent (Gallay clear 'N')**, serial rinsing in tap water and distilled and deionized water and drying are done automatically.

1.3. Sterilization of glassware

All bottles were closed with plastic or metallic caps. Flasks, cylinders and test tubes were closed with aluminium foil. Pipettes were plugged with cotton wool and put in suitable canisters. Pestles and mortars and Griffith tubes were wraped in brown paper. The glassware

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* Evans Vanodine, Eccles, Manchester, England.
** Jean Gallay and Co. Ltd., Hempstead, Herts, England.
[a] International Products Corp., Chislehurst, Kent, England.

was then sterilized either by autoclaving at 121°C for 15 minutes at a pressure of 15 lbs/sq. in. or in a hot air oven at 160°C for 2 hours, as suitable. All autoclaved glassware was dried throughly in a drying cabinet.

2. EXPERIMENTAL CHICKENS

2.1. Source

Specific-pathogen-free (SPF) light hybrid chickens were used throughout the investigation. The chickens were hatched from eggs of this Department's SPF parent flocks which have been kept in strict isolation and monitored at approximately three-monthly intervals and found free of antibodies to avian adenoviruses, avian reoviruses, Newcastle disease virus, Marek's disease virus, avian encephalomyelitis virus, infectious bronchitis virus, infectious laryngotracheitis virus, Mycoplasma gallisepticum, Mycoplasma synoviae, Salmonella gallinarum and Salmonella pullorum.

Eggs were collected twice a week and washed in an electrically operated egg washing machine (Rotomaid)* using an egg-shell disinfectant (Ryclor)**, allowed to dry on trays and stored at room temperature. The eggs were usually set within 7 days of being laid.

Eggs were incubated in a large commercial incubator with automatic hourly turning. If required for hatching,

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* Vantec Ltd., Market Drayton, Salop, England.

** Ryclor Ltd., Oakenshaw Chemical Works, Clayton-le-Moors, Accrington, England.

on the 18th day of incubation, eggs were candled for fertility and survival, and fertile embryonated eggs were transferred to a small incubator.

2.2. Housing and management

The experimental chickens were housed in an isolation unit which is a brick-walled building with nine individual pens, post-mortem laboratories and other necessary facilities. Each pen has an anteroom which links the pen to the main corridor. Both pens and anterooms are provided with negative pressure filtered air ventilation and air-tight doors. Foot baths containing iodophor based disinfectant FAM are placed at the entrance of the unit, at regular intervals along the corridor and in each anteroom. One large container full of the disinfectant is placed centrally to soak used protective clothing.

Visiting the isolation unit for observation, sampling and cleaning is strictly restricted to the person(s) involved in the experiment. All personnel visiting the unit wear rubber boots. On entering one changes into a disinfected pair of rubber boots and puts on one set of disinfected clean protective clothing (overall, mask and gloves) and collects another spare set of protective clothing in polythene bag for each pen to be visited. The visitor steps into the disinfectant baths along the corridor before entering into the anteroom of the respective pen where he puts on the

spare set of protective clothing over the first one, steps into the disinfectant foot bath and enters the pen. On leaving, the outer set of protective clothing is dipped into the disinfectant in the anteroom before being taken out in a polythene bag. All used clothing is soaked overnight in the central disinfectant bath before washing for reuse.

Chickens are kept in day old-to-death wire cages in the pens. Water is from the main supply through nipple drinkers. Electric heaters are used during the cold season and for brooding. Food is carried in double polythene bags. The outer bag is discarded and the inner bag is dipped into disinfectant after entry into the anteroom. Food and water are supplied ad libitum.

Droppings are removed regularly and sealed in double polythene bags which are dipped in disinfectant in the anteroom before being incinerated. On completion of an experiment the pen is cleaned and disinfected thoroughly. The wire cages are dismantled, soaked in disinfectant and cleaned by scraping and brushing. The air filters are also soaked in disinfectant. The anteroom and pen are fumigated with iodophor and formaldehyde. The air filters and cages are reassembled and the pen is ready for reuse.

3. CELL CULTURE

Primary chicken embryo liver (CELi) cell culture was used for virus propagation, isolation, titration and neutralization.

3.1. Culture media and supplements

M199 cell culture medium (Appendix-A) was prepared from 10X concentration and was supplemented with 10% (v/v) tryptose phosphate broth, 0.01M sodium bicarbonate, 0.014M HEPES buffer (N-2-hydroxyethyl piperazine-N'-Z- ethane sulphonic acid), antibiotics and antifungal agents (100 i.u. penicillin, 100 µg streptomycin and 2 µg amphotericin B per ml of medium). Growth medium was supplemented with 10% newborn calf serum* while maintenance medium contained 5% newborn serum.

3.2. Isolation and disaggregation of tissues

Fourteen-days old SPF chicken embryos were used for CELi cell culture preparation. The area of shell over the air space was disinfected with merthiolate** and removed with sterile instruments. The embryo was removed to a petridish and killed by cervical dislocation with the help of a pair of forceps. Embryos were opened and their livers removed leaving the gall bladders behind. The pieces of liver were collected in Dulbecco's modified phosphate buffered saline A (PBS-A) (Appendix-A) and were minced with scissors into smaller pieces. The tissues were poured into a prewarmed 100ml side-arm flask and was washed three times with prewarmed PBS-A. Then 50 ml of prewarmed

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* Gibco Ltd., Paisly, Renfrewshire, Scotland.

** E.I. Lily & Co. Ltd., Basingstoke, England.

trypsin solution (Appendix-A) was added to the tissues and incubated for 5 to 10 minutes at 37°C. Disaggregation of tissues during the incubation was facilitated by gentle stirring with a magnetic stirrer. After 5 to 10 minutes trypsinization the tissue fragments were allowed to settle and the supernatant containing cell suspension was collected in two universals containing 2 ml of ice-cold calf serum. The trypsin was inactivated by gently mixing the cell suspension with cold calf serum. This trypsin digestion was repeated for 5 to 8 times.

The cells were pelleted from the suspension by centrifugation at 800 rpm for 2 minutes. The pelleted cells from all universals were resuspended in a small volume of cell culture medium, mixed gently and pelleted again by centrifugation at 800 rpm for 2 minutes. The cells were finally resuspended in 10 ml of fresh growth medium. Viable cells were counted in a haemocytometer using 0.1% trypan blue. The final cell concentration was adjusted according to the requirement using growth medium.

3.3. Seeding of cells and incubation

Disposable tissue culture plasticware* was used for cell culture. The seeding rates of CELi cells for various tissue culture vessels were as follows: -

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*Gibco Ltd. Paisley, Renfrewshire, Scotland.

25 sq.cm. flask :8 ml ($1X10^6$ cells/ml)

80 sq.cm. flask :25 ml ($1X10^6$ cells/ml)

175 sq.cm. flask :55 ml ($1X10^6$ cells/ml)

96-well plate :0.1 ml per well ($0.3\text{-}0.5X10^6$ cells/ml)

24-well plate :1 ml per well ($0.3\text{-}0.5X10^6$ cells/ml)

12-well plate :2 ml per well ($0.6X10^6$ cells/ml)

Flasks and plates containing cells were incubated at 37°C in a humid environment with 5% CO_2 in air. Cell growth became confluent within 24 to 48 hours.

4. VIRUS AND VIROLOGICAL METHODS

4.1. Source of virus

Avian arthrotropic reovirus strain R2 was used throughout the investigation. This virus was originally isolated from an outbreak of tenosynovitis (viral arthritis) in broiler breeders (Jones et al., 1981), and had been shown to cause tenosynovitis with rupture of disital flexor tendons in SPF light breed chickens (Jones et al., 1980) and gastrocnemius tendon in commercial broiler chickens (Jones and Kibenge, 1984). The virus was plaque-purified and was used after 6 to 13 passages in CELi cell culture.

4.2. Titration of virus

The virus was titrated in 96-well microtitration plates using primary CELi cell culture as the indicator

system. A method similar to that of Grimes et al. (1976) was used.

Ten serial 10-fold dilutions of virus were made in tissue culture growth medium using bijou bottles. Fifty microliters of each virus dilution were pipetted into 4 to 8 replicate wells in a column of a microtitre plate to which 50 µl of growth medium had already been added. The last two columns of the plate contained tissue culture medium only. Then 50 µl of fresh CELi cell suspension containing $1X10^6$ cells per ml was added to each well of the plate including those in the last 2 columns which served as uninfected cell control. The cells were incubated at 37°C in a humidified incubator containing 5% CO_2 in air and examined daily with an inverted microscope for the appearance of characteristic reovirus-induced syncytial type cytopathic effects (CPE). On the 7th day of incubation the result was recorded as the number of wells showing CPE for each dilution of virus. The end-point titre as the 50% tissue culture infective dose ($TCID_{50}$) was determined by the Reed and Muench (1938) method.

4.3. Reisolation of virus

Where required in experiments, the virus was reisolated from cloacal swabs or tissue homogenates of infected birds using preformed confluent primary CELi cell culture monolayers in 24-well tissue culture plates.

Each cloacal swab was agitated vigorously in 2 ml of antibiotic broth (Appendix-A) containing 1,000 i.u. penicillin and 1 mg streptomycin per ml. Tissue samples were homogenized in a sterile pestle and mortar using a small amount of sterile sand and tissue culture medium to give an approximately 5% (w/v) suspension. Both tissue homogenates and swab suspensions were centrifuged at 750g for 10 minutes and the supernatants were collected.

Confluent CELi cell cultures in 24-well tissue culture plates were drained of growth medium and two drops of each sample were added to each of two replicate wells using a pasteur pipette. The virus was allowed to adsorb for 1 hour at 37°C, then 1 ml of fresh maintenance medium was added to each well. The cells were incubated for a further 7 days and examined daily for the appearance of any virus-induced CPE.

Any sample showing no CPE in the first passage was subjected to 3 cycles of freezing and thawing and finally vigorous pipetting to disrupt the cells. Cell culture fluids of the replicate wells were mixed together and two drops of each sample were reinoculated into fresh CELi cell culture monolayers as above. If still no CPE developed in this second passage it was subjected to another final passage before being considered as negative. The identity of the virus was confirmed from time to time by electron microscopy. Culture fluids were placed on formvar- and carbon-coated

grids and negatively stained with 4% phosphotungstic acid, pH 6.4. Examination was done using a Philips EM 201 at a magnification of 45,000X.

5. SEROLOGICAL TESTS

5.1. Agar gel precipitation test

5.1.1. Antigen

Six to seven-days old SPF chicken embryos were inoculated via the YS route with cell culture suspension of virus. The yolks of embryos that died or were still alive on the 4th day were pooled and reinoculated in 6 to 7-days old SPF chicken embryos by the YS route for a second passage. The CAMs from the dead or moribund embryos were harvested on the 4th day and ground finely with a pestle and mortar without any extra liquid being added. This material constituted the AGP antigen which was divided into small aliquots and stored frozen at 20°C until needed.

5.1.2. Agar plate

Eleven millilitres of molten agar containing 1% agar, 0.01% trypan blue, 0.01% merthiolate and 8% or 1% NaCl, for avian or mammalian sera, respectively (Appendix-A) were poured into a 90 mm plastic petri dish and allowed to solidify at room temperature. Several groups of wells were cut in each agar plate

using a template. Each group consisted of one centre well surrounded by six outer wells arranged in a daisy pattern. Wells were 3 mm in diameter and 3 mm apart. Agar plugs were removed by a Pasteur pipette connected to a vacuum pump.

5.1.3. The test

Antigen was placed in the centre well, and sera in the surrounding wells. Known positive and negative sera were incorporated as controls. Plates were incubated at room temperature in a moist chamber and read at 24 and 48 hours against a dark background with oblique illumination.

5.2. Virus neutralization test

Microneutralization tests were performed in 96-well tissue culture plate using the constant virus and varying serum dilutions (ß method).

All test sera were heated at 56°C for 30 minutes before use. Fifty microlitres of tissue culture medium were pipetted into each well of the plate, then 50 µl of each serum sample was added to two to four wells of the first column to give an initial dilution of 1:2. From this column, serial two-fold dilutions of sera were made in the subsequent wells upto column 10 using an automatic microdiluter (Dynatech Autodiluter II)*. Next, the diluted virus suspension

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* Dynatek Laboratories Inc., Alexandria, Virginia, USA.

in 50 µl volumes containing 100 $TCID_{50}$ was added to each well in columns 1 to 11. The plate was agitated for 5 minutes in a Titertek plate shaker* and incubated for 2 hours at 37°C to facilitate the neutralization of virus. Finally, 50 µl of fresh CELi cell suspension ($1X10^6$ cells/ml) was added to each well of the plate.The last two columns of the plate served as virus and cell controls, respectively. The plate was then incubated for 7 days at 37°C in a humidified atmosphere containing 5% CO_2. The cells were examined daily under a microscope for evidence of CPE. Microscopic readings were recorded on the 7th day, and the monolayers were simultaneously fixed and stained in a solution of 0.1% crystal violet in 70% alcohol for 5 minutes. The plate was washed in tap water, air dried and examined against background light. Unaffected cell sheets appeared as stained monolayers. The neutralization titre was considered to be the hightest dilution of serum producing 50% or greater inhibition of visible viral CPE and was calculated by the Reed and Muench (1938) method.

In addition to the neutralization test proper, the diluted virus suspension was simultaneously retitrated to ensure that the correct amount of virus had been used in the test.

5.3. Enzyme-linked immunosorbent assay

The general outline of the test is given here,

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* Flow Laboratories, Rickmansworth, Herts, England.

but details of the development and standardization of the technique is given in Chapter III (Part 2).

5.3.1. Antigen

Confluent monolayers of CELi cell culture grown in tissue culture flasks were infected with avian arthrotropic reovirus strain R2. After about 5 days, when maximum CPE had developed, the cells were frozen at -70°C and thawed at 37°C three times to release virus. Cellular debris was removed by centrifugation at 2000g for 20 minutes in a bench centrifuge. Supernatant fluids were centrifuged at 50,000g for 90 minutes at 4°C in an ultracentrifuge (MSE High Speed 25)*. After discarding the supernatants the pelleted virus was resuspended overnight at 4°C in a small amount of 0.05M Tris HCl-0.01M NaCl-0.001M EDTA (TNE) buffer, pH 7.8 (Appendix-A). Resuspended virus was layered onto a two-layer discontinuous caesium chloride gradient consisting of 1.5 ml of 1.6g/ml density and 5 ml of 1.25g/ml density solutions of caesium chloride in 0.05M Tris-HCl buffer, pH 7.8 (Appendix-A). The gradient was centrifuged at 90,000g for 4 hours at 4°C in a swing-out rotor of the ultracentrifuge. The diffuse band at the junction of two density solutions was collected and resuspended overnight at 4°C in 0.05M Tris-HCl buffer and repelleted by centrifugation at 50,000g for 90 minutes at 4°C.

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* MSE Scientific Instruments, West Sussex, England.

The pellet was finally resuspended overnight at 4°C in 0.05M Tris-HCl buffer to give a 100-fold concentration of original tissue culture fluid. This antigen was divided into small aliquots and stored at -70°C. The protein concentration of this antigen preparation was measured using BCA protein assay reagents*.

5.3.2. Conjugate

Rabbit-antichicken IgG (H+L) conjugated with horseradish peroxidase** was obtained commercially.

5.3.3. Diluents and washing solution

Antigen was diluted in 0.01M phosphate-buffered saline (PBS), pH 7.5 (Appendix-A). Serum and conjugate diluents and washing solutions were the same as antigen diluent but contained 0.05% Tween 20ª (PBS-Tween), while 0.015M sodium azide was added to antigen and serum diluents as preservative.

5.3.4. Substrate solution

Freshly prepared 0.06% o-phenylenediamineªª (OPD) and 0.01% H_2O_2 solution in 0.01M phosphate buffer, pH 7.0 (Appendix-A) was used as substrate.

5.3.5. Test protocol

The optimum concentrations of antigen and conjugate

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* Pierce Chemical Co., Rockford, Illinois, USA.
** Nordic Immunological Lab. Ltd., Tilburg, The Netherlands.
ª BDH Chemical Co. Ltd., Liverpool, England.
ªª Sigma Chemical Co. Ltd., Poole, Dorset, England.

were determined by checker-board titrations which are detailed in Chapter III (Part 2). Fifty microlitres of diluted antigen were added to each well of a commercially pretreated (activated) flexible poly-vinyl-chloride (PVC) microtitre plate*. After overnight incubation at 4°C, excess antigen was removed and the plate was washed three times (3 minutes each time) with washing buffer and shaken dry. Antigen-coated wells were allowed to react with appropriately diluted test sera, followed by optimally diluted conjugate, and then substrate solution. All reagents including substrate solution were added in 50 µl volume per well and incubated for 1 hour at 37°C. Between each step the excess reagents were removed by washing as before. Finally, substrate degradation was stopped by adding 30 µl of 4N H_2SO_4 per well. The absorbance of the coloured reaction product was measured at 450 nm in a microelisa reader (Titertek Uniskan)*.

All samples were tested in triplicate with each plate containing one positive and one negative reference serum.

6. IMMUNOHISTOCHEMICAL METHODS

Immunofluorescence and immunoperoxidase methods were used to demonstrate viral antigen in cell culture and tissue sections.

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* Flow Laboratories, Rickmansworth, Herts, England.

6.1. Production of reovirus-specific antisera in rabbits

CELi cell culture fluid containing reovirus strain R2 ($10^{5.8}$ $TCID_{50}$ /ml) was emulsified with an equal volume of Freund's complete adjuvant* with a homogenizer (Polytron PT10)**. This preparation was inoculated subcutaneously into six half-lop rabbits at a dose of 2 ml per rabbit. The rabbits were boosted twice by subcutaneous inoculations with 1 ml of virus suspension given three and four weeks after the primary inoculation. A test bleeding was done from the ear vein of the rabbits one week after the final inoculation and the presence of reovirus-specific antibody in serum was confirmed by AGP test. A final bleeding was done also from the ear vein 3 days after the test bleeding. The serum was separated, heated at 56°C for 30 minutes and stored in aliquots at-70°C.

6.2. Absorption of antisera with chicken tissues

Reovirus-specific rabbit antisera were absorbed with chicken liver homogenate according to the method described by Nairn (1969) with slight modification. Livers obtained from 5-weeks old SPF chickens were cut into small pieces, washed 3 times with Dulbecco's PBS-A and homogenized with an equal volume of ice-cold PBS-A in the Polytron homogenizer. The product was

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* Difco Laboratories, Detroit, Michigan, USA.

** The Northern Media Supply Ltd., Hull, England.

frozen at -70°C and thawed, centrifuged at 2000g for 10 minutes and the supernatant was discarded. The deposits were washed twice in PBS-A by centrifugation at 2000g for 10 minutes each time. Finally the homogenates were resuspended in an equal volume of PBS-A, dispensed in 1 ml volumes in microcentrifuge tubes* and centrifuged at 10,000g for 15 minutes in a micro-ultracentrifuge (Biofuge A)**. The supernatants were discarded and the tubes containing homogenates were stored at -20°C.

For the absorption, 1 ml of serum was added to 0.5 ml of tissue homogenate, mixed throughly and incubated for 1 hour at 37°C followed by overnight at 4°C. Finally, the serum was recovered by centrifuging at 10,000g for 20 minutes.

6.3. Absorption of antisera with calf serum

Reovirus-specific rabbit antisera were absorbed with insolubilized calf serum according to a modified method described by Hudson and Hay (1980).

Newborn calf serum was diluted 1:3 in PBS-A to contain 20 mg of protein per ml. A 2.5% (v/v) aqueous solution of glutaraldehyde was added dropwise to the diluted calf serum while stirring until a gel had formed. The gel was allowed to stand for 3 hours at room temperature and then dispersed with the Polytron

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* Alpha Laboratories, Eastleigh, Hampshire, England.

** Heraeus-Christ GmbH, Gipsmühlenweg, West Germany.

homogenizer. The dispersed gel was washed 5 times with PBS-A by centrifugation at 500g for 20 minutes.

Absorption was carried out by adding an equal volume of antiserum to the calf serum gel, mixing throughly and incubating for 1 hour at 37°C and overnight at 4°C. The serum was recovered by centrifugation at 500g for 20 minutes.

6.4. Indirect immunofluorescent staining of viral antigen

Infected CELi cell culture monolayers or cryostat sections of snap frozen tissues were fixed in acetone at room temperature for 10 minutes and washed for 15 minutes in 0.01M PBS, pH 7.1 (Appendix-A). Non-specific reactions were blocked by incubating the sections or cell sheets with a 1:5 dilution of normal swine serum for 30 minutes at 37°C. Swine serum was poured off and a 1:5 or 1:10 dilution of reovirus-specific rabbit antiserum was applied to the sections or cell monolayers. Preimmunization rabbit serum was used instead of antisera as control. After 1 hour incubation at 37°C in a moist atmosphere, unattached antibodies were removed by agitation in PBS for 30 minutes. Excess washing buffer was removed by blotting slides around the sections or cell monolayers. A 1:50 dilution of commercial swine-antirabbit IgG conjugated with fluorescein isothiocyanate*, was applied and incubated for 1 hour at 37°C. The sections or cell monolayers

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* Nordic Immunological Lab. Ltd.,Tilburg,The Netherlands.

were washed again as before and mounted with coverslips using buffered glycerol containing 0.1% p-phenylene-diamine (Appendix-A). These preparations were examined under a Leitz Ortholux fluorescence microscope equipped with incident UV/blue illumination.

6.5. Immunoperoxidase staining of viral antigen

Peroxidase-antiperoxidase (PAP) and avidin-biotinylated enzyme complex (ABC) methods were used to demonstrate viral antigen in cell culture monolayers and paraffin-embedded sections. Below is given the general procedure for conducting immunoperoxidase tests, the principle of which is given in Chapter III (Part 1). Most reagents for PAP and ABC methods of staining were obtained commercially as test kits*. Staining was done according to manufacturer's instruction with necessary modifications.

Acetone-fixed infected cell culture monolayers or deparaffinized and rehydrated tissue sections were washed for 15 minutes in 0.05M Tris buffered saline (TBS), pH 7.6 (Appendix-A). Endogenous peroxidase activity was quenched by treating the sections or cell monolayers with 3% H_2O_2 for 5 minutes. After washing for 15 minutes in 3 changes of TBS, excess fluid was removed from the slides by blotting with tissue paper. Non-specific reactions were blocked by incubating the

* PAP Kit: Miles Scientific, Slough, England.
ABC Kit: Sera-Lab Ltd., Sussex, England.

sections or monolayers for 20 minutes at room temperature with normal swine or goat serum for the PAP and ABC methods, respectively. The normal serum was poured off and various dilutions (1:50 to 1:1000) of reovirus-specific rabbit antiserum were applied. Following 1 hour incubation at 37°C the unattached antibody was removed by washing as above. Reagents used in the next two steps in the PAP method were swine-antirabbit IgG and peroxidase-rabbit antiperoxidase complex, both for 20 minutes at room temperature. In ABC method biotinylated goat-antirabbit IgG and avidin-biotinylated peroxidase complex were used for 30 minutes and 45 minutes at room temperature, respectively. Between each step the sections or monolayers were washed in TBS as above.

The antigen-antibody reaction was visualized by colour development with either of two substrate solutions using the following methods:

a) 3,amino, 9,ethylcarbazole (AEC) and H_2O_2 solution (both supplied with the PAP Kit) were mixed and filtered immediately before use according to manufacterur's instruction, applied to the sections or monolayers and incubated for 20-40 minutes in the dark at room temperature. After brief rinsing in tap water, preparations were counterstained with Mayer's haematoxylin, and mounted in molten glycerol jelly and overlaid with a coverslip.

b) Alternatively, freshly made 0.05% 3,3'diaminobenzidine tetrahydrochloride (DAB) in 0.1M Tris buffer, pH 7.2 containing 0.01% H_2O_2 (Appendix-A) was applied to the sections or monolayers and incubated for 3 to 7 minutes at room temperature in the dark. After a brief wash in tap water, tissues were counterstained with Mayer's haematoxylin, dehydrated in graded alcohol, cleaned in xylene and mounted in DPX as in conventional histological methods.

Preimmunization rabbit serum was used as a negative control replacing immune serum.

7. HISTOLOGICAL METHODS

Tissue blocks were fixed in Bouin's solution (Appendix-A) for 6 to 24 hours and then rinsed in and transfered to 70% alcohol. Blocks were trimmed, dehydrated and cleaned in an automatic tissue processor and embedded in paraffin wax. Sections were cut at a thickness of 4 to 5 microns with a microtome and stained with Mayer's haematoxylin and eosin (HE) according to conventional methods.

Chapter III

DEVELOPMENT OF SOME IMMUNOLOGICAL TECHNIQUES USED IN SUBSEQUENT REOVIRUS PATHOGENESIS STUDIES

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Chapter III

DEVELOPMENT OF SOME IMMUNOLOGICAL TECHNIQUES USED IN SUBSEQUENT REOVIRUS PATHOGENESIS STUDIES

The pathogenesis studies on avian reovirus infections described in subsequent chapters in this thesis required techniques for (i) tracing the distribution of reovirus in the cells of selected tissues by immunohistochemical means at intervals after infection, and (ii) detecting and quantitating the humoral antibody response. For the former, immunofluorescence and immunoperoxidase staining were used, and for the latter ELISA was selected. This chapter describes the adaptation of these immunological techniques for the specific requirements of avian reovirus investigation.

Part 1

IMMUNOHISTOCHEMICAL DEMONSTRATION OF AVIAN REOVIRUS IN CELL CULTURE

1. INTRODUCTION

The development of the fluorescent antibody (FA) technique (immunofluorescence) by Coons et al. (1941) made it possible for the first time to visualize an antigen in tissue sections. In the original direct FA technique, the tissues containing antigen were treated with antibody conjugated with a fluorochrome, usually fluorescein isothiocyanate (FITC). However, this technique suffered from the disadvantage of having to conjugate each antiserum used to detect different antigens. This difficulty was largely overcome and the sensitivity of the method enhanced by the development of an indirect FA technique. In this method, an unlabelled primary antibody is allowed to bind with the antigen in the specimen and then a FITC-conjugated appropriate species-specific anti-gamma globulin is applied to react with the primary antibody. The double layer results in considerable amplification and stronger staining intensity. Moreover, the species-specific labelled antibody can be used

with a range of primary antisera raised in that species against different antigens.

Immunofluorescent staining for viruses may be done on cell culture and tissue smears, but for pathogenesis experiments the most useful preparations are cryostat sections of snap-frozen tissues, although immunofluorescence can also be used on paraffin sections. The major disadvantages of immunofluorescent staining have been the lack of permanent preparations, due to fading during examination and storage, and also the need for a fluorescence microscope equipped with UV/blue illumination and suitable filters.

A useful alternative, however, is the use of enzymes instead of fluorochrome for labelling antibodies. Various enzymes such as horseradish peroxidase, alkaline phosphatase, glucose oxidase and ßgalactosidase can be used to label antibodies, but horseradish peroxidase has been the most popular one. Enzyme labels are visualized by addition of appropriate substrates and there is the advantage that the coloured, insoluble and permanent reaction products, precipitated at the site of antigen localization, can be observed by conventional light microscopy.

The original immunoenzyme techniques (Nakane and Pierce, 1966) were based on the same principles as those in direct and indirect immunofluorescence and produced comparable results. However, the

development of peroxidase-antiperoxidase (PAP) (Sternberger et al., 1970) and avidin-biotinylated enzyme complex (ABC) (Hsu et al., 1981) techniques has further increased the sensitivity of enzyme-immunohistochemical methods. In these techniques the primary antibody is linked by a secondary antibody to an enzyme-anti-enzyme immune complex (PAP) or a purely chemical macromolecular complex containing an enzyme (ABC). Thus a greater amplification of the reaction can be achieved. The principles of various immunohistochemical techniques are presented diagrammatically in Fig. III.1.

Immunohistochemical techniques are now widely used for immunodiagnosis and pathogenesis studies of many viral diseases (Kurstak and Morisset, 1974). A direct FA test has been used to demonstrate avian reovirus in cell culture (Kawamura and Tsubahara, 1966) and in chicken tissues (Menendez et al., 1975b; Jones and Onunkwo, 1978). An indirect FA test using chicken-antireovirus serum and anti-chicken conjugate has been described by Ide (1982) and Adair et al. (1987) to demonstrate reovirus in cell culture monolayers. However, the use of labelled antichicken globulin would not be satisfactory for certain chicken tissues as the conjugate may directly bind to any chicken immunoglobulin already present.

Therefore, it appeared necessary to develop suitable and sensitive immunohistochemical techniques

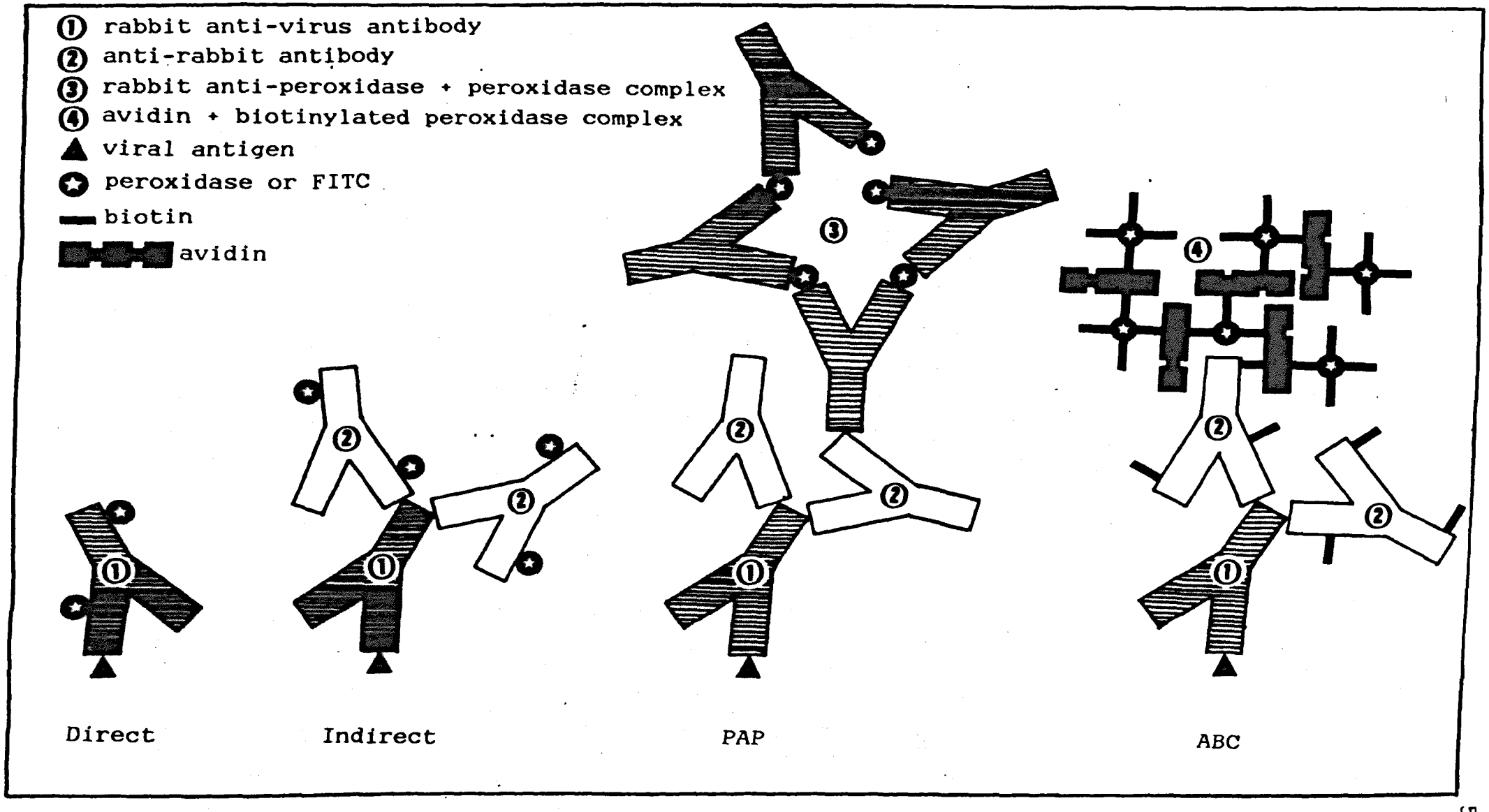

Fig. III.1. Schematic representation of the principles and methods of immunohistochemical techniques for detecting viral antigen

for detecting avian reovirus in tissue sections for pathogenesis study. This section describes the development and standardization of an indirect immunofluorescence and PAP and ABC methods of immunoperoxidase (IP) tests using rabbit-antireovirus serum.

2. MATERIALS AND METHODS

2.1. Preparation of infected cell culture monolayers

Reovirus-infected CELi cell culture monolayers were used to develop and standardize FA and IP techniques. One drop (approximately 0.05 ml) of CELi cell suspension ($1X10^6$ cells/ml) was placed in each well of a clean and sterile teflon-coated 12-well slide* and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. After 24 hours, when confluent monolayers had developed, the growth medium was removed and one drop of reovirus suspension in maintenance medium containing approximately 10^5 $TCID_{50}$ of virus was added to each well. After incubation for 24 hours at 37°C the cells were examined frequently for CPE. When reovirus-induced syncytia were first visible, the monolayers were fixed in acetone for 10 minutes at room temperature after a brief rinse in PBS-A. Following fixation the slides were either immediately transferred

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* Flow Laboratories, Rickmansworth, Herts, England.

to appropriate washing buffer prior to staining or were air-dried and stored at 4°C in a sealed polythene bag until ready for use.

2.2. Anti-reovirus serum

The methods of producing reovirus-specific rabbit antiserum and its absorption with chicken liver homogenate and calf serum are given in Chapter II (Sections 6.1, 6.2 and 6.3, respectively).

2.3. Selection of optimum conditions for staining

The optimum working dilutions of rabbit anti-reovirus serum and anti-rabbit fluorescent conjugate for the indirect FA test were determined by checkerboard titration using serial dilutions of antiserum (1:5, 1:10, 1:15 and 1:20) and conjugate (1:25, 1:50, 1:75 and 1:100). Incubations were performed for 30 to 60 minutes either at room temperature or at 37°C.

For IP staining all reagents except primary antiserum were used according to manufacturer's instructions. Various dilutions (1:25 to 1:2000) and incubation times (20 minutes to 1 hour at room temperature or at 37°C, or overnight at 4°C) were used for the primary antiserum. Substrate incubation time also varied, such as 20 to 40 minutes for AEC and 3 to 10 minutes for DAB solutions.

2.4. Staining methods

Standardized procedures for the indirect FA staining and the PAP and ABC methods of IP staining are given in Chapter II (Sections 6.4 and 6.5, respectively).

3. RESULTS

For both methods initial trials with unabsorbed primary reovirus antiserum resulted in very high background reactions. However, the absorption of the antiserum with chicken liver homogenate and calf serum effectively reduced the background staining.

The optimum working dilutions of the primary antiserum and conjugate for indirect FA staining were found to be 1:10 and 1:50, respectively. Incubation for 1 hour at 37°C for both antiserum and conjugate provided the most intense staining.

A 1:100 dilution of the primary antiserum was found to be optimum in the PAP and ABC methods of IP staining. Incubation for 1 hour at 37°C or overnight at 4°C produced equally good results.

After indirect FA staining viral antigen appeared as small granular to larger globular fluorescence in the cytoplasm of CELi cells (Fig. III.2). Similar

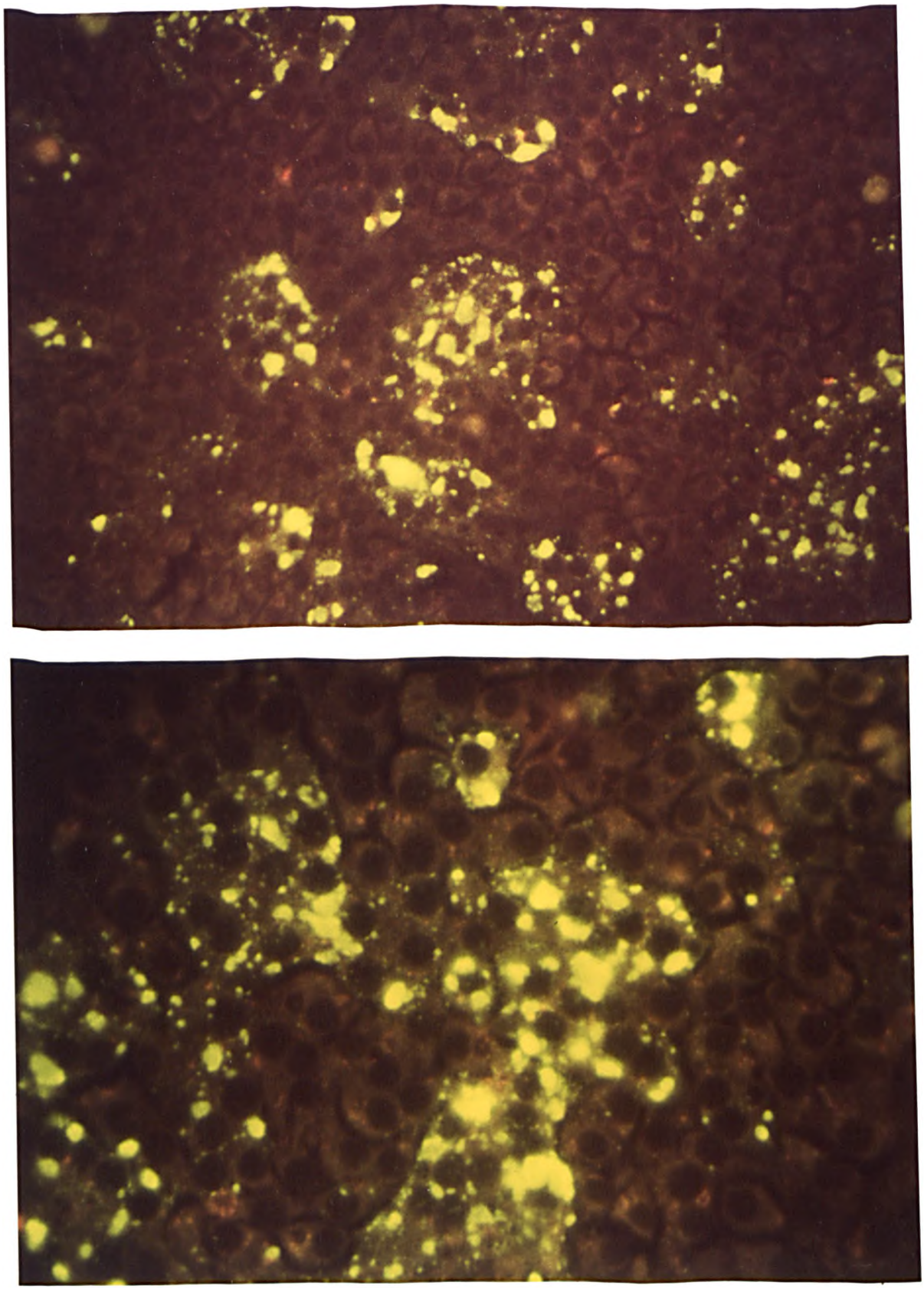

Fig.III.2. Indirect immunofluorescence staining of reovirus-infected chicken embryo liver cell culture at 16 hours p.i. Viral antigens are intracytoplasmic and appear as large globules or smaller granules (top X423, bottom X667).

cytoplasmic staining was observed after the IP methods (Fig III.3), which appeared as brick-red or dark brown precipitates with AEC and DAB chromogen, respectively.

4. DISCUSSION

Both FA and IP staining appeared to be suitable for demonstrating viral antigen in infected cell culture preparations.

The antiserum used in this study was raised in rabbits by inoculating CELi cell culture fluid containing virus; this was used after clarification by low-speed centrifugation but without further purification. As a result, the reovirus-specific antibodies were associated with several unwanted antibodies, particularly those directed to CELi cell components and the calf serum supplement of the medium. These unwanted antibodies produced very high background staining in the initial trials of FA and IP staining. Therefore, the antiserum had to be absorbed with chicken liver homogenate and calf serum before being used for staining.

Immunoperoxidase staining has the advantages over immunofluorescence that a permanent preparation is obtained which can be examined by ordinary light

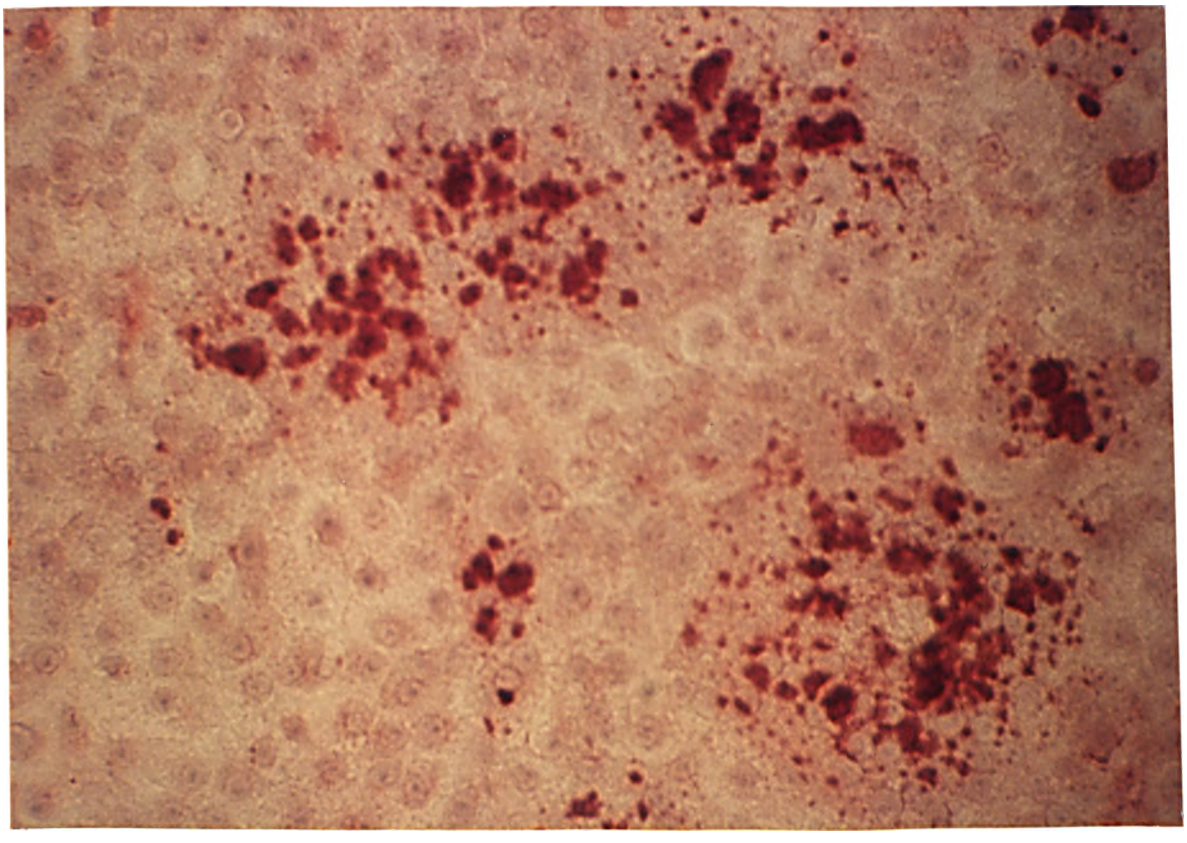

Fig.III.3a. The PAP method of immunoperoxidase staining of reovirus-infected chicken embryo liver cell culture at 16 hours p.i. Viral antigens appear as brick-red irregular intracytoplasmic precipitates (AEC chromogen, lightly counterstained with haematoxylin, X423).

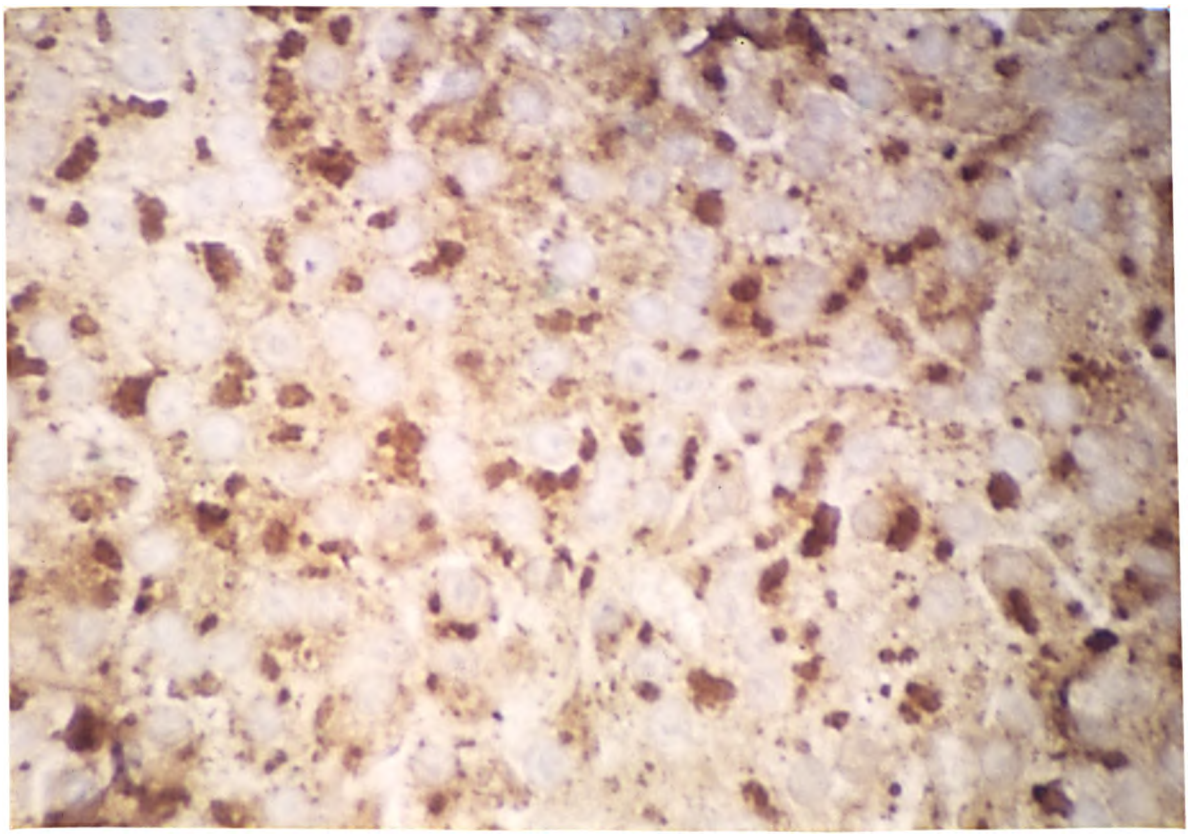

Fig.III.3b. The ABC method of immunoperoxidase staining of reovirus-infected chicken embryo liver cell culture at 16 hours p.i. More uniformly distributed viral antigens appear as dark brown granular intracytoplasmic precipitates (DAB chromogen, lightly counterstained with haematoxylin, X677).

microscopy. In the present study, the IP technique appeared to be more sensitive than FA for detecting reoviral antigen in cell culture since for the former, the same primary antiserum could be used at a dilution 10 times higher than that required for the latter. However, in reovirus-infected cell cultures, the IP staining did not appear to provide any better definition of antigen localization as compared with the FA.

From these trials on reovirus-infected cell cultures, it would seem that the immunohistochemical techniques could be an important tool for studying the pathogenesis of avian reovirus infection.

Part 2

AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR MEASURING ANTIBODY TITRE AGAINST AVIAN REOVIRUS IN A SINGLE DILUTION OF SERUM

1. INTRODUCTION

Commonly used serological tests for detecting antibodies to avian reovirus include AGP and VN tests. Because of the limited sensitivity of AGP and prolonged cell cultivation in VN tests, neither of these methods appears to be suitable for certain experimental purposes such as vaccine evaluation or pathogenesis studies and rapid, routine seroprofiling of chicken flocks for reovirus antibody.

Following the introduction (Engvall and Perlmann, 1971) and microtitre plate adaptation (Voller et al., 1974) of ELISA, its potential has been tested for serodiagnosis of numerous infectious diseases of man and animals.

ELISA results are usually presented in two forms: either as antibody titre determined by conventional end-point dilution, or as absorbance values measured in a single dilution of serum. The former method provides easily communicable test results but is not as economic as the latter method which, however, suffers from difficulties in interpretating results.

A third approach is to transform the absorbance values measured in a single serum dilution into ELISA antibody titres using a standard curve. Several methods of transforming absorbance values into antibody titres have been described (de Savigny and Voller, 1980; van Loon et al.,1981; Malvano et al.,1982; Snyder et al., 1983; Brigs et al.,1986; Hatfield et al., 1987), but no single method has been generally accepted.

Slaght et al. (1978) described an ELISA for detecting antibodies to avian reovirus in chicken serum. Part 2 of this chapter describes the standardization and adaptation of an ELISA for reovirus antibody and also compares several methods of predicting ELISA antibody titres using a single dilution of serum.

2. MATERIALS AND METHODS

The basic protocol of the ELISA procedure and the details of antigen preparation, conjugate, substrate solution, diluents and washing solution are given in Chapter II (Section 5.3).

2.1. Determination of optimum dilutions of antigen and conjugate

The optimum working dilutions of antigen and conjugate were determined by several checkerboard titrations. Serial dilutions of antigen in PBS and

of conjugate in PBS-Tween were tested using reovirus-positive and negative reference sera while keeping all other parameters constant as described in the ELISA protocol (Chapter II, Section 5.3.5). The dilutions of antigen and conjugate which had provided the highest absorbance ratio of the positive to the negative sera were selected as the optimum dilutions.

2.2 Antisera

Reovirus-positive reference serum comprised a pool of three antisera raised in mature SPF chickens. Two subcutaneous inoculations of reovirus strain R2 were given one month apart and the sera were collected one week after the second inoculation. Other reovirus-positive sera used for the construction and evaluation of standard curves (see below), were obtained from SPF chickens at intervals after a single oral or foot-pad inoculation of the virus. Reovirus-negative reference serum was a pool of sera collected from 90 mature SPF chickens.

2.3. Comparison of various antigen coating methods

Two reo-positive sera, one negative serum pool and one serum blank (diluent only) were tested in plates coated with antigen by different methods. Antigen was diluted in distilled water (Slaght et al., 1978), or 0.05M carbonate-bicarbonate buffer, pH 9.6 (Appendix-A) (Voller et al., 1976) or NaOH solution,

pH 13 (Miers et al., 1983), or 0.01M PBS, pH 7.5. Activated PVC plates containing antigen were incubated either for 1 hour at 37°C or overnight (18 hours) at 4°C. In addition to these, rigid polystyrene micro-titre plates were pretreated with foetal calf serum (FCS) and glutaraldehyde and coated with antigen diluted in distilled water as described by Slaght et al. (1978). All other steps were essentially the same as described under the ELISA protocol (Chapter II, Section 5.3.5).

2.4. Prediction of antibody titre in single serum dilution

Twenty reo-positive sera having a wide range of antibody levels when tested in a preliminary ELISA were selected to derive standard curves for converting absorbances measured in single dilutions of serum into antibody titres.

2.4.1. End-point titration

All 20 sera were titrated in serial dilutions at 0.5 $\log_{10}$ intervals. Absorbance values were multiplied by a correction factor (Snyder et al., 1983) to minimize day-to-day fluctuations in absorbance values. The correction factor for a particular plate was obtained as a ratio between expected absorbance (average of 10 separate determinations) and observed absorbance of a reference positive serum at 1:100

dilution which was incorporated in each plate. The positive-negative threshold (PNT) baseline was determined as the mean plus twice the standard deviation of corrected absorbance values of negative serum pool in a range of dilutions (1:100 to 1:100,000) obtained from three separate assays. To determine the end-point titre, $\log_{10}$ serial absorbances of each serum were plotted against $\log_{10}$ corresponding dilution reciprocals. The point at which absorbance curve intersected the PNT baseline was considered as the end-point titre.

2.4.2. Computational methods

Standard curves and corresponding prediction equations were derived for predicting antibody titres in three single dilutions, i.e. 1:100, 1:316 and 1:1000, from five different forms of absorbance values, namely, (i) raw absorbance, (ii) corrected absorbance (as described above), (iii) specific absorbance (absorbance of negative reference serum was subtracted from that of the sample), (iv) sample/positive ratio (a ratio of the absorbances of sample and positive reference serum), and (v) sample/negative ratio (a ratio of the absorbances of sample and negative reference serum). Separate regression analyses were performed using $\log_{10}$ absorbances of 20 sera in all three dilutions and for all five forms of absorbances. The titre prediction equation for each form of

absorbance in a particular single serum dilution was as follows:

$$\text{Log}_{10}\text{Titre} = (\text{Log}_{10}\text{Absorbance} - \text{Y intercept})/\text{slope}.$$

2.5. Comparison of various titre-prediction methods

Five reovirus-positive sera having different antibody levels, as determined by a preliminary ELISA not included in standard curve analyses, were selected for testing different titre prediction methods. End-point titres of all 5 sera were determined as described above. The absorbance values of these 5 sera and the reo-positive and reo-negative reference sera were then determined in three single dilutions (1:100, 1:316 and 1:1000) on two different occasions. Then the titres were predicted from absorbance values for all 5 sera in all 3 single dilutions using 5 different prediction methods as described above. Predicted titres were compared with original end-point titres. A deviation of the predicted from the end-point titres within a range of 0.3 $\log_{10}$ or a two-fold dilution was considered to be acceptable.

3. RESULTS

3.1. Optimum dilutions of antigen and conjugate

Results of the checkerboard titrations are given in Fig. III.4. The purified 100-fold concentrated

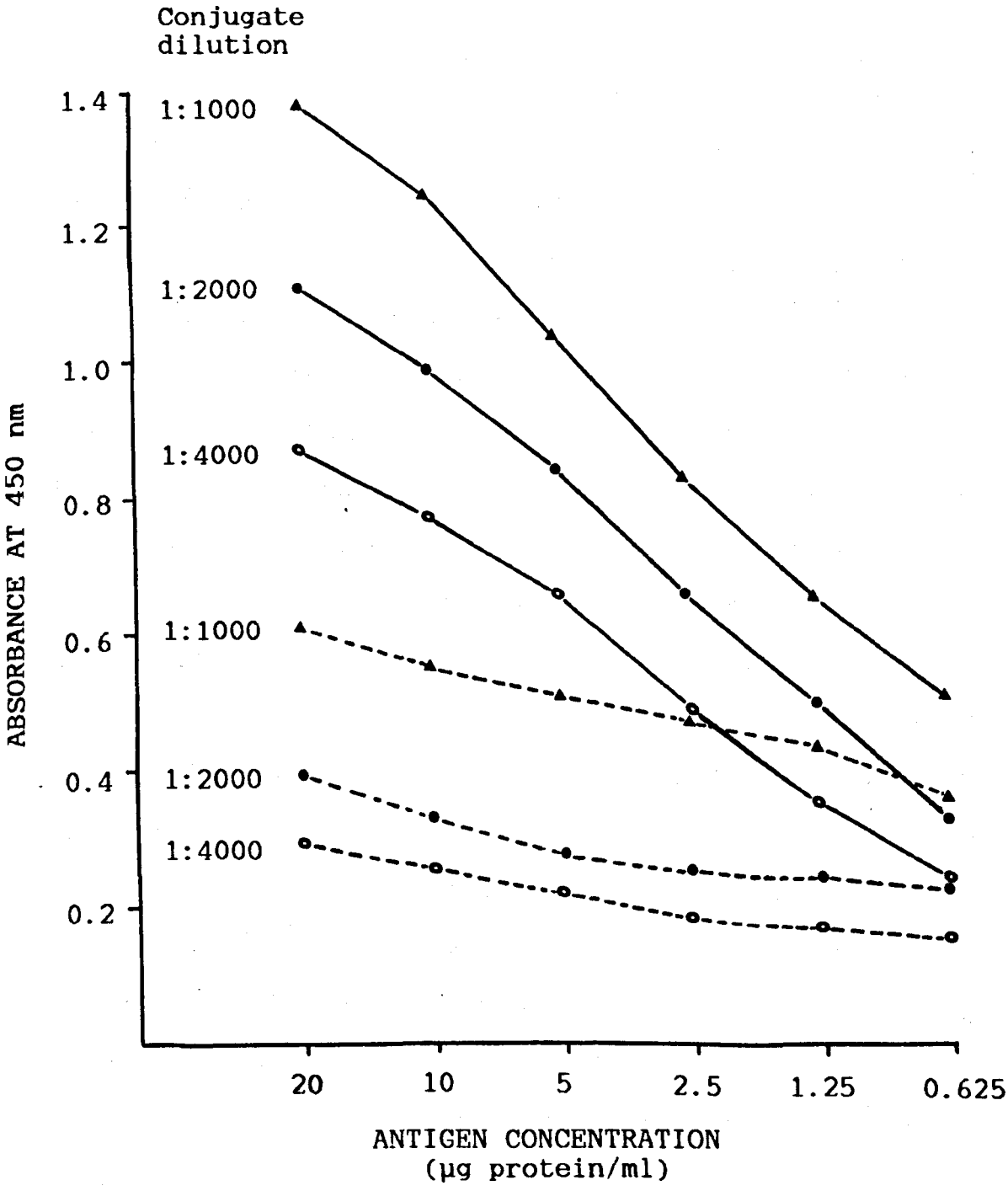

Fig. III.4. Absorbances of a reovirus-positive (solid lines) and a reovirus-negative (broken lines) reference sera in a checkerboard ELISA for reovirus antibody with serial dilutions of antigen and three selected dilutions of conjugate.

antigen had a protein concentration of 0.5 mg/ml. A 1:100 dilution of antigen containing 5 µg of protein/ml and 1:2000 dilution of conjugate were found to be optimum.

3.2. Effects of various antigen-coating methods

Effects of various antigen-coating methods on the ELISA absorbance values of two reo-positive sera, one reo-negative serum pool and serum blank are shown in Table III.1. Following overnight antigen-coating at 4°C both reo-positive sera reacted equally well with antigen diluted in carbonate buffer and PBS, but, the nonspecific absorbance of negative serum was lower with the latter. When distilled water or NaOH was used as antigen diluent, the absorbance values of one positive serum were very low; moreover, the reo-negative serum gave very high non-specific absorbance with antigen diluted in NaOH. Following a short period of antigen-coating, i.e. 1 hour at 37°C, the effects of all four antigen diluents were almost the same as those which occurred following overnight antigen coating except that the absorbance values were comparatively lower in all cases.

When antigen, diluted in distilled water, was allowed to bind to FCS-glutaraldehyde pretreated plate, the absorbance values of both positive and negative sera and as well as serum blank were elevated as compared with those in untreated plates.

Table III.1. Effects of various antigen-coating methods on ELISA absorbance values of two reovirus-positive sera, one reovirus-negative serum pool and a serum blank

Plate	Flexible poly-vinyl-chloride								Polystyrene	
Pretreatment of plate	Commercially pretreated (activated)								FCS+G[a]	----
Antigen coating time and temp.	Overnight (18h) at 4°C				1h at 37°C				Until dry, at room temp.	
Antigen coating buffer	Carbonate buffer pH 9.6	PBS pH 7.5	Distilled water	NaOH solution pH 13	Carbonate buffer pH 9.6	PBS pH 7.5	Distilled water	NaOH solution pH 13	Distilled water	Distilled water
Reovirus-positive sera A/85	0.99[b]	0.97	0.82	1.10	0.86	0.70	0.57	0.78	1.07	0.79
R/5	0.80	0.83	0.48	0.59	0.42	0.38	0.23	0.30	0.75	0.57
Reovirus-negative serum pool SPF(90)	0.44	0.28	0.33	0.79	0.38	0.16	0.22	0.67	0.39	0.25
Serum blank	0.11	0.12	0.15	0.10	0.10	0.13	0.15	0.10	0.34	0.16

a. FCS+G, foetal calf serum and glutaraldehyde
b. Mean absorbance values of 3 separate assays measured at 450 nm. Serum dilution 1:100

3.3. Sensitivity and stability of ELISA for reovirus antibody

Dose-response absorbance curves of strong, moderate and weak reo-positive sera and a reo-negative serum pool are shown in Fig. III.5. The PNT baseline absorbance was calculated and appeared to be 0.3 over the range of serum dilutions between 1:100 and 1:100,000.

Intra-assay and between-run coefficients of variation (CV) observed in this ELISA system for reovirus antibody were always below 10% and 15%, respectively.

3.4. Regression statistics for predicting titres

The results of $\log_{10}$ titre versus $\log_{10}$ absorbance regression analyses using 20 sera are presented in Table III.2. One of these 15 regression analyses, corrected absorbance versus end-point titre, is presented graphically in Fig. III.6. The correlation coefficient was very high in all 3 serum dilutions and for all 5 forms of absorbances, with a minimum correlation coefficient (r) of 0.88. In all methods a higher correlation between the titre and absorbance was observed with higher serum dilutions. The slopes of regression lines were similar in all cases except for the specific absorbance method. This was due to the fact that the arithmetical subtraction of a fixed absorbance value of the negative reference serum from the absorbances of positive sera did not cause equal reductions in their log-transformed data.

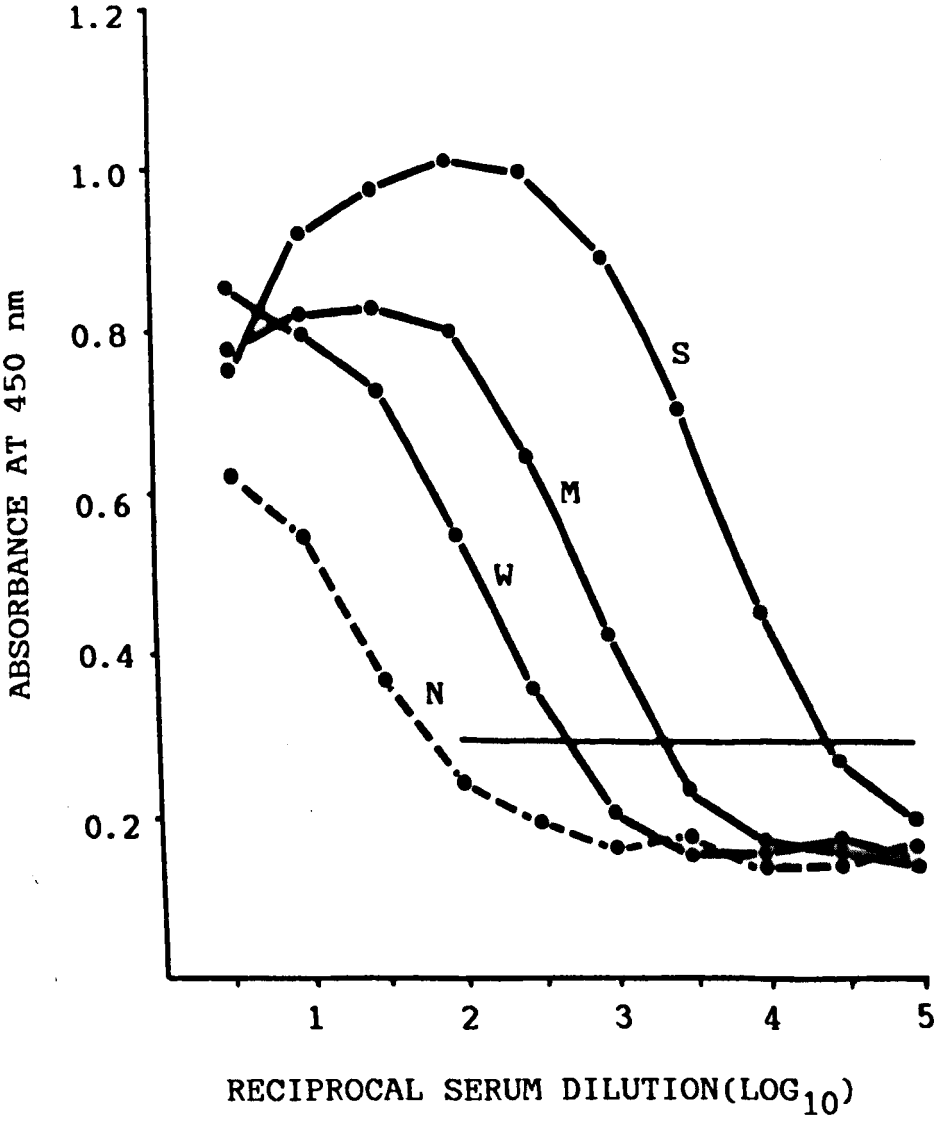

Fig. III.5. Absorbance profiles of strong (S), moderate (M) and weak (W) reovirus-positive sera and one reovirus-negative (N) serum pool in serial dilutions. Horizontal line represents positive-negative threshold baseline (see Section 2.5.1) at corresponding dilutions.

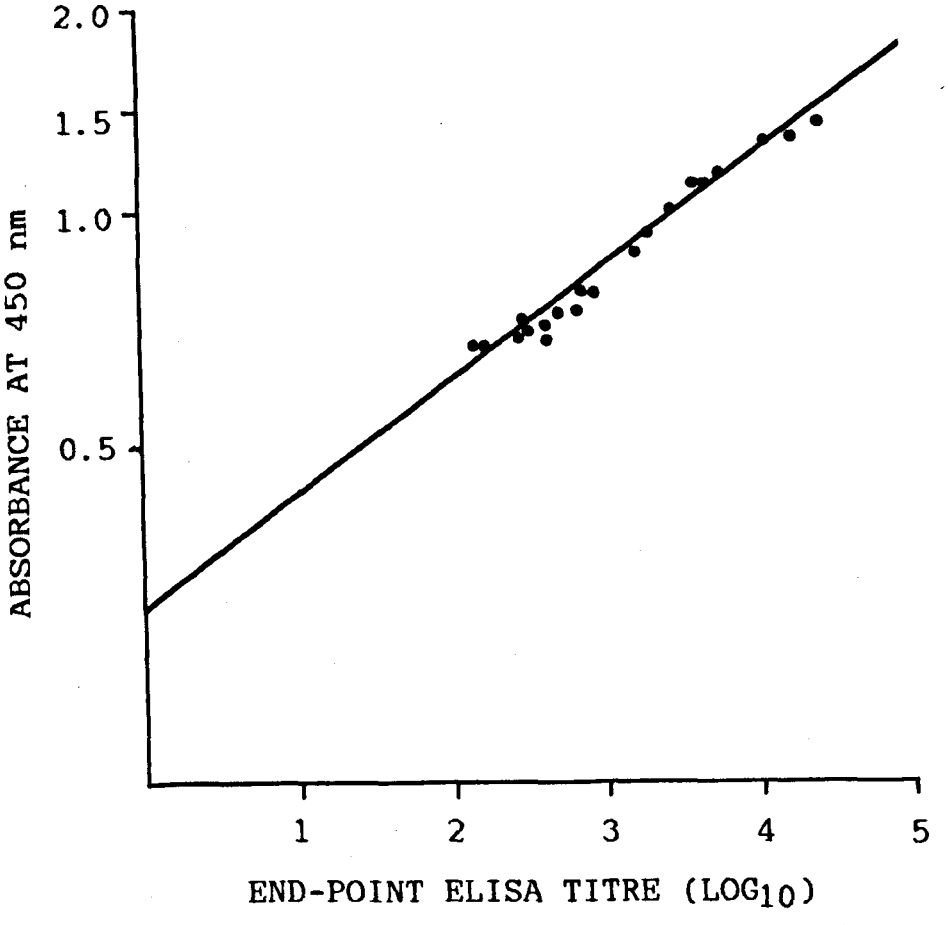

Fig. III.6. Relationship between corrected absorbance in ELISA of a 1:1000 dilution of sample and its end-point titre for twenty chicken sera containing reovirus antibody (r=0.98).

Table III.2. Regression statistics for predicting ELISA antibody titre to reovirus by different methods from absorbance values measured in a single dilution of serum (n=20)

'X' versus 'Y'	Statistics	Working serum dilution		
		1:100	1:316	1:1000
Log_{10} titre	r	0.92	0.98	0.98
vs.	y intercept	-1.11	-1.52	-1.77
Log_{10} raw absorbance	Slope	0.27	0.37	0.40
Log_{10}titre	r	0.93	0.97	0.98
vs.	y intercept	-0.81	-1.26	-1.50
Log_{10} corrected absorbance	Slope	0.20	0.30	0.34
Log_{10} titre	r	0.88	0.92	0.97
vs.	y intercept	-1.93	-3.13	-3.86
Log_{10} specific absorbance	Slope	0.45	0.76	0.90
Log_{10} titre	r	0.92	0.96	0.97
vs.	y intercept	-0.77	-1.12	-1.36
Log_{10} sample/positive ratio	Slope	0.20	0.29	0.34
Log_{10} titre	r	0.92	0.96	0.98
vs.	y intercept	-0.25	-0.60	-0.77
Log_{10} sample/negative ratio	Slope	0.20	0.30	0.36

3.5. Stability of various titre-prediction methods

The performance of various methods in predicting ELISA antibody titres from absorbance values of 5 different sera measured in 3 single serum-dilutions (1:100, 1:316 and 1:1000) are shown in Table III.3. When titres were predicted from raw absorbances, corrected absorbances or sample-positive ratios, the percentage of acceptable predictions(where the predicted titre was within the range of a two-fold dilution, above or below, of the end-point titre) gradually increased with the increase in serum dilution. In all dilutions, the highest number of acceptable predictions could be made from the corrected absorbances and sample-positive ratios. On the other hand, sample/negative ratios provided the minimum number of acceptable predictions.

Antibody titres of 5 different sera determined by end-point dilution or predicted form absorbances in 1:1000 dilution are shown in Table III.4. All the titres predicted from corrected absorbances or sample/positive ratios were very close to the end-point titres. The titres predicted from raw absorbances and specific absorbances were mostly acceptable, but those obtained from sample/negative ratios were largely unacceptable.

Table III.3: The performance of various methods in predicting ELISA antibody titres to reovirus from absorbance values measured in a single dilution of serum

Methods	Working serum dilution		
	1:100	1:316	1:1000
Raw absorbance	50[a]	60	90
Corrected absorbance	70	90	100
Specific absorbance	70	60	70
Sample/positive ratio	70	90	100
Sample/negative ratio	40	30	30

a. Percentage of acceptable predictions, out of 10 made for 5 different sera. A predicted titre within the range of a two-fold dilution, above or below the end-point titre was considered to be an acceptable prediction.

Table III.4. Reovirus antibody titres of 5 different sera determined by different methods from absorbance values measured in a single dilution (1:1000) of sera

Serum identity	End-point titre	Titre predicted from				
		Raw absorbance	Corrected absorbance	Specific absorbance	Sample/ positive ratio	Sample/ negative ratio
7/2	3,020	5,495*	3,890	6,607na	4,074	8,511na
		2,042*	3,236	4,365	3,467	9,772na
3/2	3,715	5,012	3,467	5,888	3,715	7,586na
		1,862	3,090	4,074	3,020	8,511na
R/5	6,918	6,607	5,012	6,607	5,129	5,012
		3,715	6,457	6,166	6,918	19,953na
10/6	22,909	22,387	20,893	14,791	20,893	45,709
		15,849	27,154	13,183	31,623	112,201na
1/6	39,811	23,442	30,909	15,136na	29,119	48,978
		17,782na	42,658	14,125na	42,658	131,826na

* Titres predicted from absorbance values measured in two separate assays.

na. Predicted titre not acceptable as it varied more than a two-fold dilution range of end-point titre.

4.DISCUSSION

An indirect ELISA for detecting antibody to avian reovirus has been described by Slaght et al. (1978). In this laboratory, this method did not appear to be readily adaptable without further modifications. FCS and glutaraldehyde pretreatment of microtitre plate did not reduce the non-specific reactions, as claimed by Slaght et al. (1978). On the contrary, this treatment increased the background noise. High non-specific reactions following FCS and glutaraldehyde pretreatment were also observed by Miers et al. (1983). They recommended as an alternative, the use of NaOH solution, pH 13, as antigen diluent. However, in the present study, use of NaOH solution as antigen diluent also appeared to be unsatisfactory because of high non-specific reaction. The use of more conventional carbonate-bicarbonate buffer, pH 9.6 or PBS, pH 7.5 as antigen-diluents produced acceptable results, and moreover, PBS further reduced the non-specific reaction with negative serum. Therefore, 0.01M PBS, pH 7.5 was used as the antigen diluent in the present ELISA system. It should be noted that the optimum concentrations of antigen and conjugate were determined by checkerboard titrations using PBS as antigen diluent. It was not determined whether the use of other antigen-coating methods in checkerboard titrations would have influenced the selection of optimal conditions for the test.

The peroxidase substrate o-phenylenediamine is usually diluted in a buffer at around pH 5.0, allowed to react for a short time (5-15 minutes) and the absorbance is read at 492 nm. However, at this pH the substrate solution is very sensitive to light and the colour development occurs very quickly. Thus very strict standardization of timing is essential to get reproducible results. In the present study, this problem was largely overcome by diluting the substrate in phosphate buffer, pH 7.0, incubating for 1 hour and reading the absorbance at 450 nm (Slaght et al, 1978). At this higher pH substrate solution is less sensitive to light and the enzyme action is slow.

The ELISA system described in this chapter appeared to be sensitive and reproducible. Intra-assay and between-run CV were within the acceptable range of 10 - 15% (McLaren et al., 1981).

The principle of predicting the ELISA antibody titre in a single dilution of serum using a constructed dose-response standard curve has been generally accepted. However, disagreement exists as to the choice of computational methods and the forms of absorbances used for constructing the standard curves and predicting titres.

In the present study linear regression analyses provided very good correlations between log-transformed data of end-point titres and various forms of absorbance

values measured in a single dilution of serum. However, among the three single dilutions used in this study, the 1:1000 dilution provided the highest correlation in all five methods. The relatively poor correlation coefficient obtained with a 1:100 dilution might be due to a prozone reaction of some positive sera as suggested in Fig. III.5. The same phenomenon has also been observed by de Savigny and Voller (1980).

Despite very high correlation, the prediction methods did not appear to be equally stable when they were tested for titration of 5 different sera. The sample/negative ratio method (Briggs et al., 1986) was the least successful. This method assumes that in day-to-day variations, both positive and negative sera will fluctuate by the same ratio, which is not true, as the absorbance values of negative and positive sera represent the non-specific and specific reactions, respectively.

The specific absorbance methods, where the absorbance value of a negative serum is subtracted from that of positive sample (Snyder et al., 1983), relies on two assumptions. Firstly, the absorbance of a negative serum represents the internal background noise of the positive serum, and secondly, day-to-day variations in absorbance values are due to the fluctuations in background noise only. These two assumptions are only partly justified as the specific absorbance of a positive serum could also be affected by day-to-day

variation. Probably for these reasons, this method did not appear to be as stable as others.

The raw absorbance method depends heavily on the stability of absorbance values of positive serum, which is practically impossible to obtain.

The corrected absorbance method (Snyder et al., 1983) and sample/positive ratio method assume that the absorbance values of all positive sera would fluctuate in the same ratio. This assumption is largely true within limits imposed by minor variations such as: slight changes in the pH of buffers, freshness of the substrate solution, age of antigen and conjugate, and time and temperature of incubation. The maximum stability in titre-prediction was observed by the corrected absorbance and sample/positive ratio methods. Similar results obtained by these two methods were due to the use of the absorbance of the same positive reference serum for calculating both the corrected absorbance and sample/positive ratio. However, the corrected absorbance method would appear to be more versatile in storing the data in the forms of both corrected absorbance and predicted titre.

Because of the higher linear correlation between titre and absorbance, and better stability in prediction, a serum dilution of 1:1000 should be used for predicting titre. However, if the absorbance value of any serum measured at 1:1000 dilution appears to be below the PNT baseline absorbance, a lower

dilution, such as 1:100 should be used for predicting the titre. Any titre below 1:100 should be considered as negative for diagnostic purposes.

The application of ELISA for evaluating humoral antibody responses to reovirus in chickens and the correlation between ELISA and VN antibody titres will be described in Chapter VI.

Chapter IV

EARLY PATHOGENESIS OF EXPERIMENTAL REOVIRUS INFECTION IN CHICKENS

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Chapter IV

EARLY PATHOGENESIS OF EXPERIMENTAL REOVIRUS INFECTION IN CHICKENS

1. INTRODUCTION

It would appear from the literature reviewed in Chapter I that, with the exception of tenosynovitis (viral arthritis), the relationship between avian reoviruses and various disease conditions from which they had been isolated is not yet fully established. Reovirus-induced tenosynovitis, a persistent viral infection with granulomatous lesions, is confined mainly to the hock joints and leg tendons of chickens. However, a wide distribution of the virus in various tissues during the early stage of infection has been reported (Menendez et al., 1975b; Ellis et al., 1983 and Kibenge et al., 1985). Apart from this, very little is known about the early events in the pathogenesis of avian reovirus infection, such as the portal of entry, site of primary replication and the route and sequence of spread of the virus within the host.

This chapter describes the results of some virological, immunohistochemical and electron microscopic studies on the early events of experimental reovirus infection in chickens.

2. MATERIALS AND METHODS

2.1. Experiment 1. Virological study

2.1.1. Experimental design

Thirty-three 1-day-old chicks were infected orally with 0.3 ml ($10^{5.3}$ $TCID_{50}$) of virus suspension per bird. Another group of 12 chicks from the same hatch was used as uninfected controls. These two groups were housed separately.

Birds were sampled at the following intervals: 2,6,12 and 24 hours and 2,3,4,5,7,9 and 12 days post infection (p.i.). Three infected and 2 uninfected control birds were sampled on each occasion. Each bird was bled by cardiac puncture and swabbed from the cloaca before being killed by cervical dislocation.

2.1.2. Collection of tissues for virological study

Carcasses were opened aseptically and pieces of tissues were collected from the following organs for virus isolation and titration: a) approximately 1.5 cm long portions from various parts of the intestine, including descending and ascending duodenum, middle jejunum, posterior ileum, ileo-caecal junction and rectum, and also bursa of Fabricius; b) pieces of extra-intestinal tissues including liver, pancreas, spleen, heart, kidney and hock-joint. Special care was taken to avoid tissue-to-tissue contamination, and separate

sets of sterile instruments were used for collecting different tissues. Collection of intestinal tissues was not attempted until all other extra-intestinal tissues had been collected. The portions of intestine were emptied of their contents by gentle squeezing with forceps to minimize the effects of the varying amounts of intestinal contents on the result of virus titration. On every occasion, tissues obtained from each organ of all birds belonging to the same group (i.e., infected and uninfected) were pooled, weighed and stored at -20°C until used.

2.1.3. Virus isolation and titration

A total of 280 pools of tissues were collected in this experiment. The tissues were homogenized with pestles and mortars or Griffith tubes using a small amount of sterile sand and tissue culture medium 199 without serum to give 10% (w/v) suspensions. All suspensions were centrifuged for 10 minutes at 750g in a bench centrifuge and the supernatants were collected. The amount of virus in the supernatant was titrated on freshly prepared CELi cell culture. The method was basically the same as that described in Chapter II (Section 4.2), except that the dilutions were made either at 2-fold or 0.5 $\log_{10}$ intervals instead of 10-fold. End-point titres, calculated as $TCID_{50}$ per 50 µl of suspension by the Reed and Muench (1938) method, were normalized to $TCID_{50}$ per gram of tissues.

The amount of virus present in the sera was also titrated by the same method and the titres were normalized to $TCID_{50}$ per ml of serum. The original samples of those found negative for virus in titration were subjected to 3 passages in preformed CELi cell culture for virus isolation as described in Chapter II (Section 4.3.). Isolation of virus from cloacal swabs was also done by the same method.

2.2. Experiment 2. Immunohistochemical study

2.2.1. Experimental design

Fourteen 1-day-old chicks were infected orally with the same dose of virus as used in Experiment 1. Two chicks each time were sampled at 6 and 12 hours, and 1,2,3 and 5 days p.i. They were killed by cervical dislocation and opened immediately following aseptic procedure. Small pieces of tissues were collected from the duodenum, jejunum, ileum, bursa of Fabricius, liver, spleen, heart, kidney and hock joints for immunofluorescence and immunoperoxidase study as described below.

2.2.2. Snap-freezing of tissues for immunofluorescent staining

An aluminium foil cup, measuring about 1 cm in diameter, was partially filled with a special embedding medium (O.C.T. compound)* and a small piece of tissue

* Miles Scientific, Slough, England.

was placed and oriented in the embedding medium. The cup was completely filled with medium and the tissue was then snap-frozen by immersing the cup in liquid nitrogen for about 30 seconds. The embedded and frozen tissues were put into self-sealing polythene bags and stored immediately at -70°C until required for use.

2.2.3. Fixation of tissues for immunoperoxidase staining

Small pieces of tissues were fixed either in Bouin's fluid for 6-24 hours or in formol-sublimate (Appendix-A) for 12-24 hours. Fixed tissues were embedded in paraffin and sectioned at 4 microns thickness using conventional histological methods. Mercuric salt was removed from formol-sublimate fixed tissues before commencing immunoperoxidase staining by treating the sections with 0.5% iodine in 70% alcohol for 4 minutes followed by 2.5% sodium thiosulphate.

2.2.4. Sectioning of frozen tissues

Frozen tissues were sectioned at -20°C on a cryostat*. After removing the aluminium foil cover, the frozen tissue block was attached to a chuck with a drop of O.C.T. compound and secured in position by freezing immediately with dichlorofluoromethane aerosol spray (Cryo-jet)**. Sections were cut at 5 microns thickness, mounted on slides, air-dried and fixed in acetone for 10 minutes at room temperature.

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* Slee Medical Equipments Ltd., London, England.
** BDH Chemicals Co. Ltd., Liverpool, England.

2.2.5. Immunohistochemical staining methods

Details of the production of reovirus-specific antiserum and its absorption with chicken liver homogenate are given in Chapter II (Section 6). The procedures for indirect FA and ABC method of IP staining used in this study were similar to those described in Chapter II (Section 6) and Chapter III. However, in addition to the described IP technique, deparaffinized and rehydrated sections were treated with a prewarmed solution of 0.1% trypsin and 0.1% $CaCl_2$ in 0.05M Tris-HCl buffer, pH 7.8 for 10-15 minutes at 37°C to expose antigen which might have been masked by overfixation.

2.3. Experiment 3. Electron microscopic study

2.3.1. Experimental design

A group of 16 one-day-old chicks were infected orally with the same dose of virus as used in Experiment 1. Two chicks were sampled on each occasion at the following intervals: 3,6,12,24,36,48,72 and 96 hours p.i.

2.3.2. Collection and processing of tissues for electron microscopy

Chicks were killed by cervical dislocation and opened immediately. Small pieces of tissues were collected aseptically as quickly as possible from the

duodenum, jejunum, ileum and bursa of Fabricius. Tissue samples were fixed in Karnovsky's solution (Appendix-A) for 2 to 4 hours and then transferred to PBS-A. Tissues were postfixed in 1% aqueous osmium tetroxide, dehydrated and embedded in epoxy resin. Thin sections were cut on an ultramicrotome, stained with uranyl acetate and lead citrate and examined with a Philips EM 201 electron microscope.

3. RESULTS

3.1. Virological findings

The results of virus titrations from enteric tissues including the bursa of Fabricius are presented in Fig. IV.1. As early as 2 hours after infection a small amount of virus, which almost certainly represented the virus of the inoculum, was found throughout the intestine and in the bursa of Fabricius. Viral titres in different parts of the intestine started to rise by 12 to 24 hours after infection and reached their peaks at 2 or 3 days p.i. The titres of virus in the posterior part of intestine (ileum to rectum) started to fall at 3 or 4 days p.i. while in the upper part of the intestine (duodenum and jejunum) titres maintained their peaks until day 5 p.i. All parts of the intestine still contained considerable amounts of virus at day 12 p.i., when the experiment was

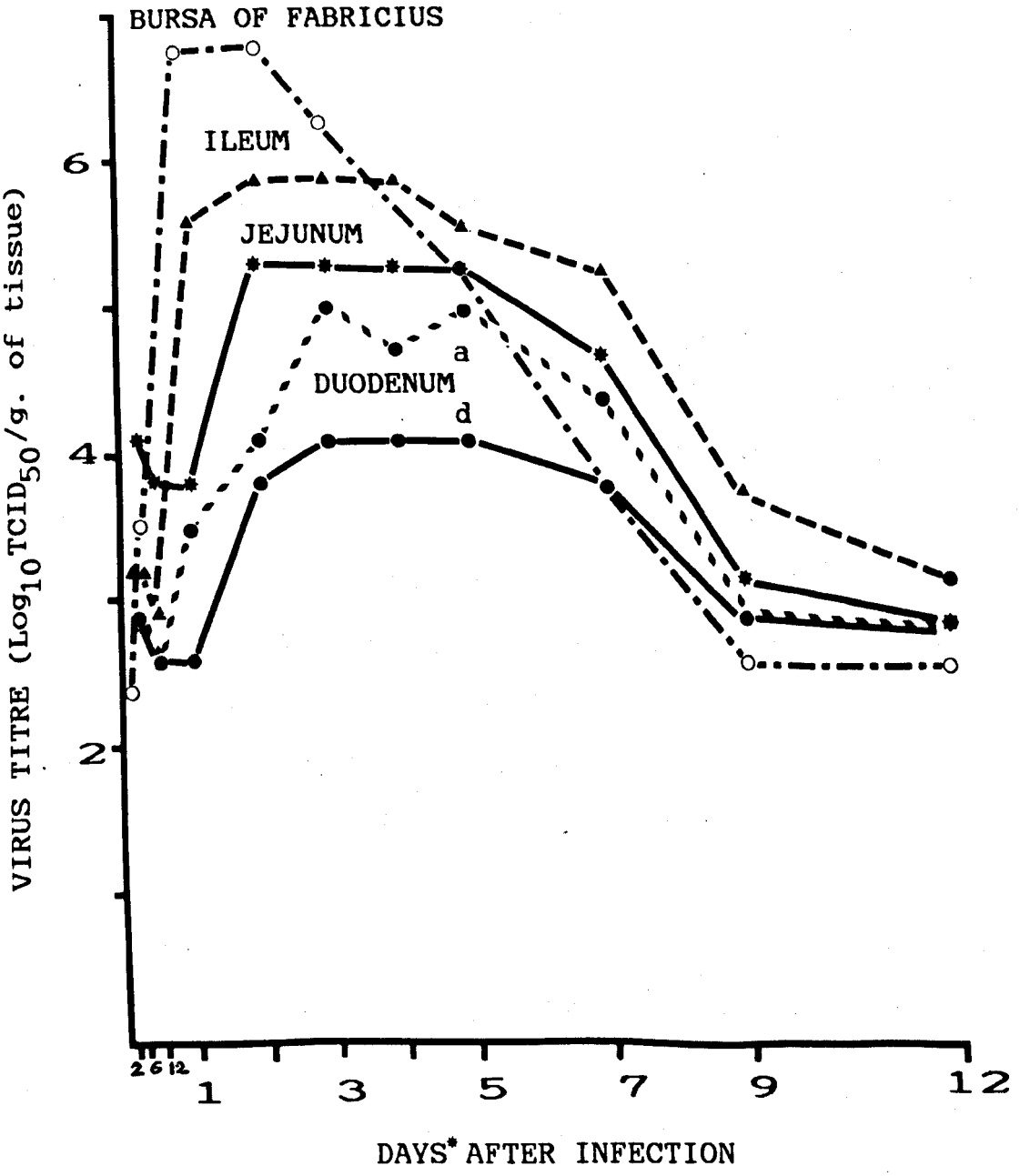


Fig. IV.1. Titre of virus in different parts of intestine and bursa of Fabricius of chickens at various time after oral infection with reovirus (5.3 $log_{10}TCID_{50}$ per bird) at 1-day of age.
a and d stand for the ascending or descending part of duodenum, respectively.

*First 3 samplings were done at 2, 6 and 12 hours p.i.

terminated. The highest titre of virus in this study ($10^{6.8}TCID_{50}$ /g of tissue) was observed in the bursa of Fabricius as early as 1 day after infection and the high titres were maintained for a further 1 or 2 days before they gradually declined. Viral titres of the ileo-caecal junction and rectum are not shown in the figure to avoid overcrowding of lines, but they were similar to those of ileum except that the rectum had a very high titre of virus ($10^{6.8}TCID_{50}$ /g of tissue) on day 2 and 3 p.i.

The results of virus isolation and titration from extra-intestinal tissues and serum are presented in Table IV.1. The earliest detectable virus was in the liver and kidney where trace amounts were found 6 hours after infection. However, from most tissues virus was recorded from 12 hours p.i. (pancreas, heart) or 24 hours p.i. (liver, spleen, kidney), and virus was found in all of the tissues examined at day 2 p.i. Consistent levels of virus were found in liver, pancreas, spleen and kidney between 1 and 5 or 7 days p.i. The presence of amounts of virus which could be titrated ($\leq 10^{2.6}$ $TCID_{50}$ /g of tissue) in the heart and hock joint was delayed by 2-3 days relative to other tissues, and a high titre was recorded for the former at 12 days p.i. when all other tissues except kidney contained trace amounts of virus.

Virus was isolated from serum between 2 and 5 days p.i.; the highest titres being observed on days 3 and 4 p.i.

Table IV.1. Titre of virus in various extra-intestinal tissues and serum at different times after oral infection of day-old chicks with reovirus R2 (5.3 $\log_{10}$ $TCID_{50}$ /bird)

Tissues	Time after infection										
	2h	6h	12h	1d	2d	3d	4d	5d	7d	9d	12d
Liver	-	*[1]	-	3.2[a]	4.7	4.4	4.1	4.4	3.2	*[1]	*[3]
Pancreas	-	-	3.8	3.5	3.8	3.5	3.2	3.5	3.5	*[1]	*[1]
Spleen	-	-	-	2.9	4.1	4.1	4.4	4.4	3.5	2.9	*[1]
Heart	-	-	2.9	-	*[1]	*[1]	2.9	3.2	3.2	3.5	4.7
Kidney	-	*[1]	-	3.8	3.2	3.2	3.8	*[1]	2.6	*[1]	-
Hock joint	-	-	-	-	*[1]	*[1]	5.0	3.2	2.9	3.8	*[1]
Serum	NP	NP	NP	-	*[1]	2.9	2.9	*[1]	-	-	-

a. Virus titre ($\log_{10}$ $TCID_{50}$ /g of tissue)

* Virus present (but less than 2.6 $\log_{10}$ $TCID_{50}$ /g of tissue). Superscript numbers indicate the number of passage(s) required for the isolation of virus.

NP. Not performed.

-. Negative for virus after 3 blind passages.

Cloacal swabs were positive for virus on every occasion throughout the experiment (not shown in table).

No virus was detected in any tissues, cloacal swabs or sera of uninfected control birds.

3.2. Immunohistochemical findings

The results of immunohistochemical staining are shown in Table IV.2. Viral antigen was detected in the duodenum, jejunum and bursa of Fabricius between 1 and 3 days after infection, maximum specific fluorescence being observed at days 1 and 2 p.i. Virus-specific fluorescence was seen in the ileum on one occasion only, at 1 day p.i. Specific fluorescence was not observed in the sections of heart and kidney, which were examined at days 3 and 5 p.i. only. The specificity of the weakly defined, finely granular fluorescence seen in the liver and spleen was inconclusive. In the hock joint, all chondrocytes gave strong, probably non-specifc, fluorescence, but specific fluorescence of viral antigen could not be identified.

In the intestine, viral antigen appeared as globular intracytoplasmic fluorescence in a few epithelial cells and lamina propria (Fig. IV.2). In the bursa of Fabricius, virus-specific intracytoplasmic fluorescence was observed in many epithelial cells (Fig. IV.3). At day 1 p.i. the fluorescence was largely confined to the bursal epithelium, but on day 2 and 3 some cells in the lymphoid follicles near the surface were also positive for viral fluorescence.

Table IV.2. Immunohistochemical demonstration of viral antigen in various tissues after oral infection of day-old chicks with reovirus R2.

Tissues	Time after infection					
	6h	12h	1d	2d	3d	5d
Duodenum	-	-	F(++) P(+)	F(++) P(+)	F(+) P(+)	P(+)
Jejunum	-	-	F(++) P(+)	F(++) P(+)	F(+) P(+)	P(+)
Ileum	-	-	F(+)	-	-	-
Bursa	-	-	F(+++) P(+++)	F(++) P(++)	F(+) P(++)	P(++)
Liver	NP	NP	F(±)	F(±) P(±)	F(±) P(±)	P(+)
Spleen	NP	NP	-	F(±)	-	-
Kidney	NP	NP	NP	NP	-	-
Heart	NP	NP	NP	NP	-	-
Hock joint	NP	NP	NP	NP	-	-

'F' and 'P' stand for positive fluorescence and peroxidase staining, respectively.

Number of '+' indicates the intensity of positive staining, and '-' indicates no specific staining.

'NP' denotes not performed.

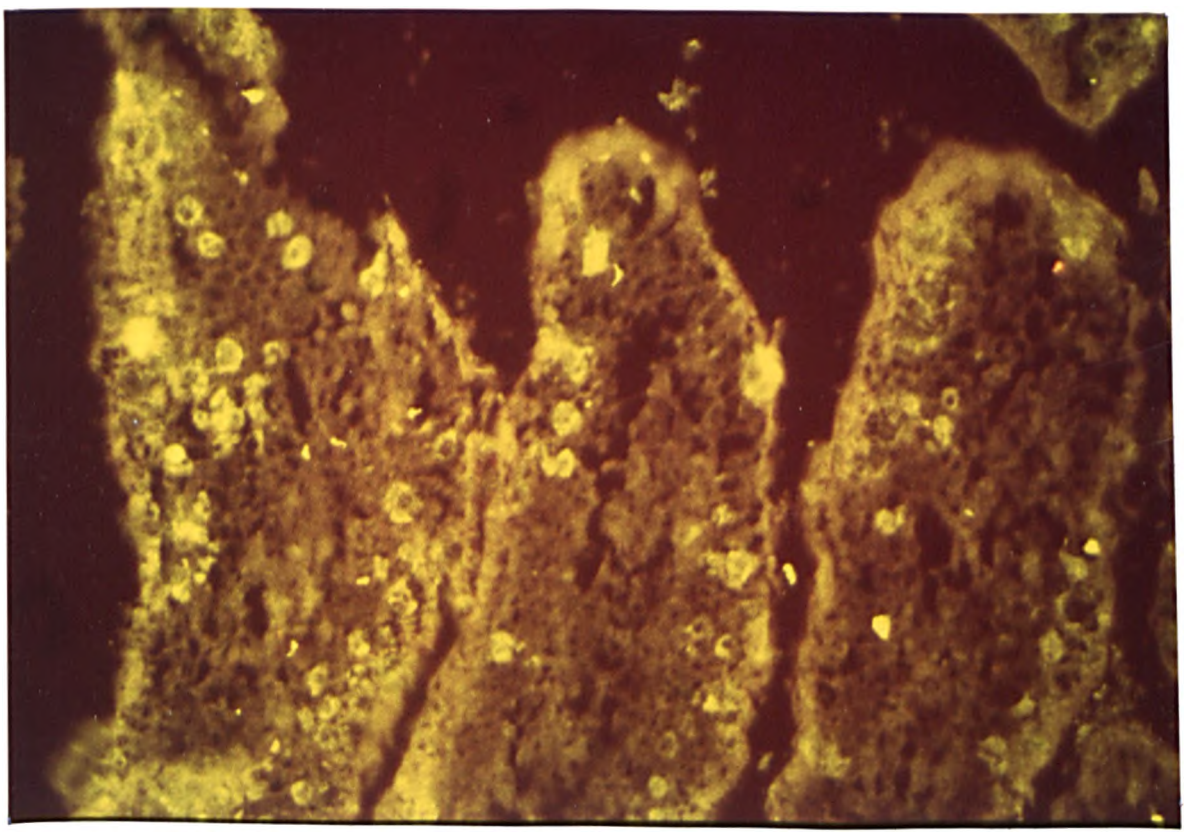


Fig.IV.2. Indirect immunofluorescence staining of intracytoplasmic reoviral antigens in the epithelial cells and lamina propria of jejunal villi at day 1 p.i. (X423).

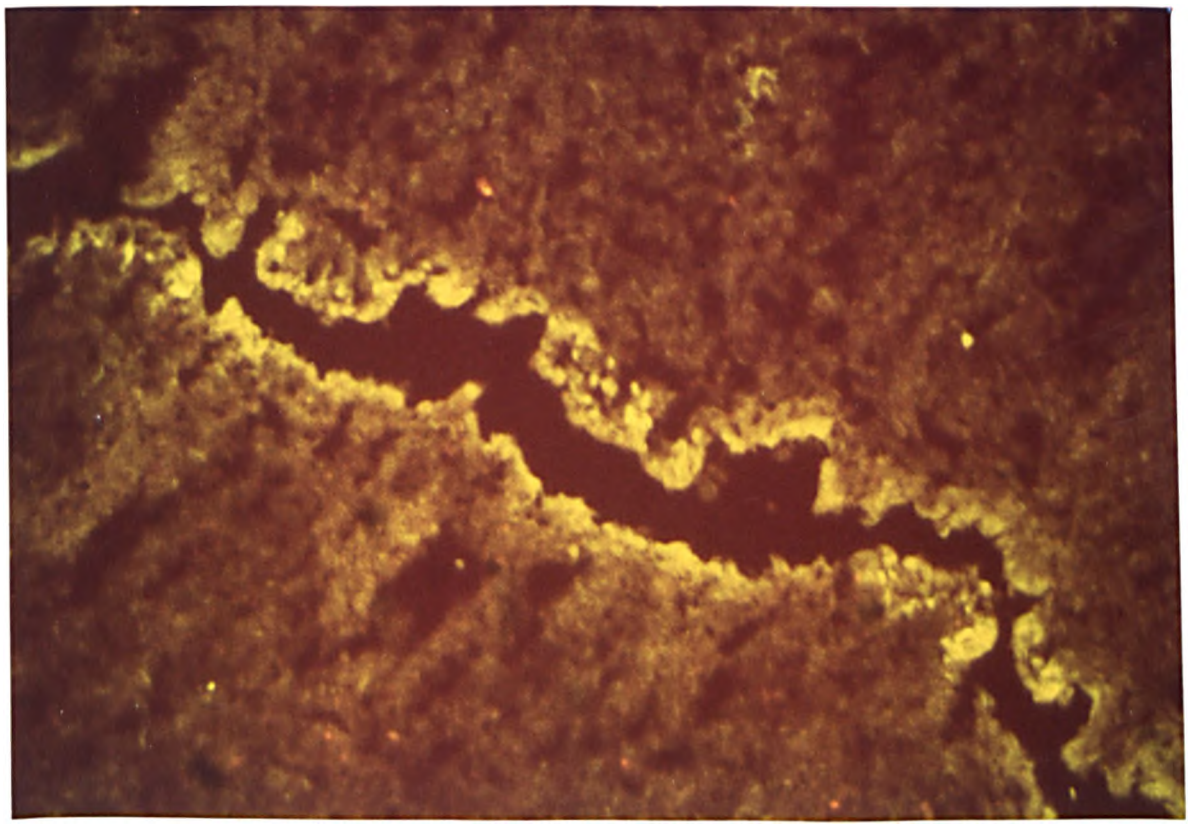
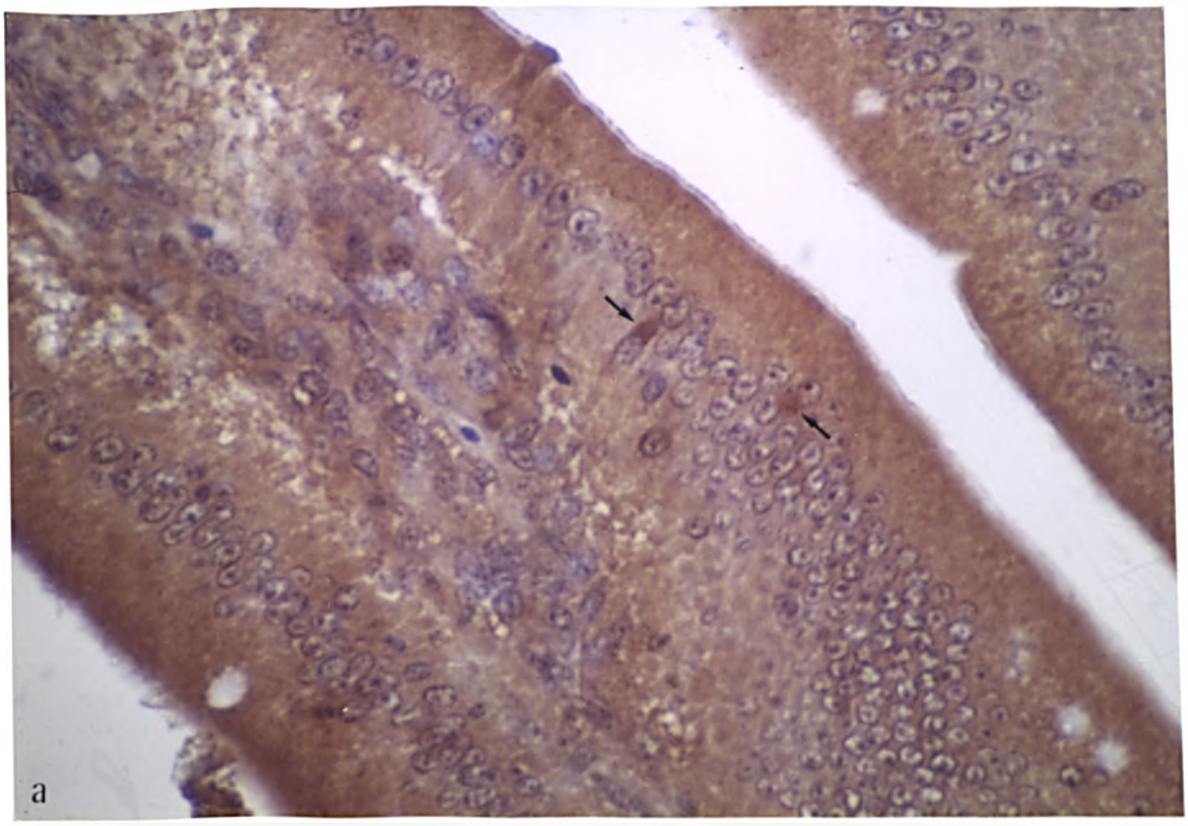


Fig.IV.3. Indirect immunofluorescence staining of reoviral antigen in the epithelial lining of the bursa of Fabricius at day 1 p.i. (X423).

By the ABC method of IP staining viral antigen was detected in the bursa of Fabricius, duodenum and jejunum from day 1 p.i. and in liver from day 2 p.i. until the end of the experiment (day 5 p.i.). Virus-specific peroxidase staining appeared as dark brown granular intracytoplasmic precipitate with DAB chromogen. In the bursa of Fabricius positive staining was observed in the cells of the bursal epithelium (Fig. IV.4) and also in the lymphoid follicles. In the duodenum and jejunum, a positive reaction was seen in a very small number of epithelial cells and in the lamina propria (Fig. IV.5). In the liver positive staining was observed in the hepatocytes which sometimes had a foamy appearance due to vacuolation of the hepatic cytoplasm (Fig. IV.6). Viral antigen could not be detected in spleen, heart, kidney or hock joint by the IP staining.

3.3. Electron microscopic findings

A consistent finding throughout the experiment was the presence of numerous viral particles between the microvilli of the epithelial brush border in all regions of the intestine examined. However, their presence appeared to be restricted to certain epithelial cells while the surfaces of adjacent enterocytes were completely free of viral particles (Fig. IV.7). Virions were seen inside the enterocytes in smooth-surfaced vesicles close to the surface from 12 hours p.i. Single



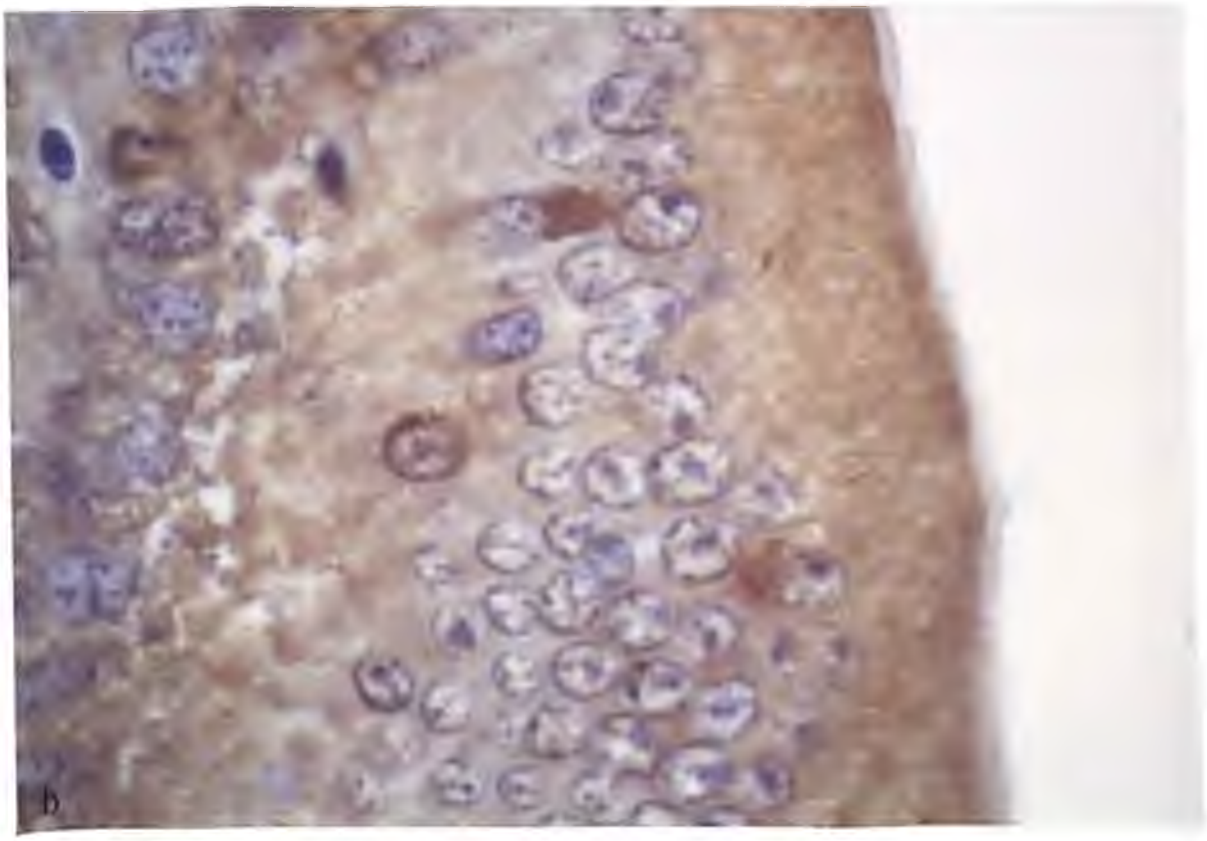


Fig.IV.4a. Immunoperoxidase staining of reoviral antigen in the cytoplasm of epithelial cells (arrows) in the jejunum at day 1 p.i. (ABC method, DAB chromogen, haematoxylin counterstain, X667).

b. Details of the above at higher magnification showing diffuse cytoplasmic distribution of antigen (X1,693).

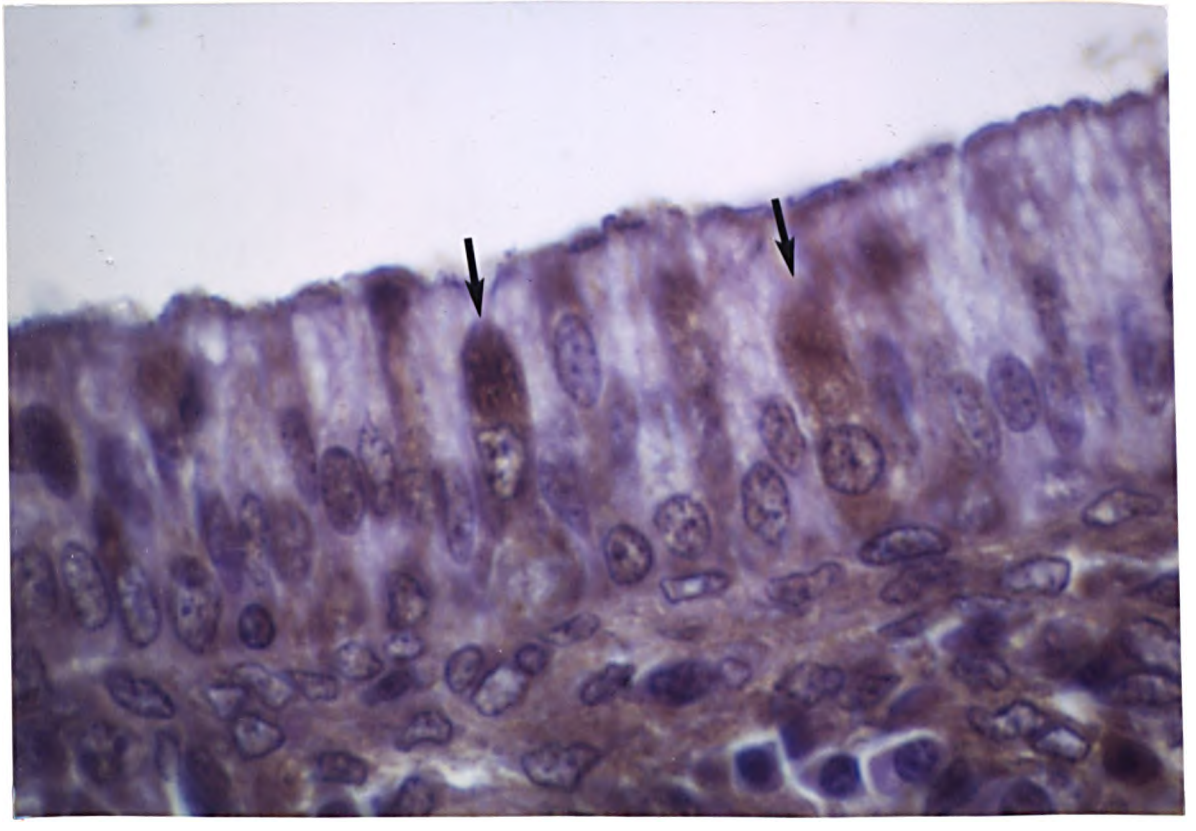


Fig.IV.5. Immunoperoxidase staining of reoviral antigen in the cytoplasm of epithelial cells (arrows) in the bursa of Fabricius at day 1 p.i. (ABC method, DAB chromogen, haematoxylin counterstain, X1,693).

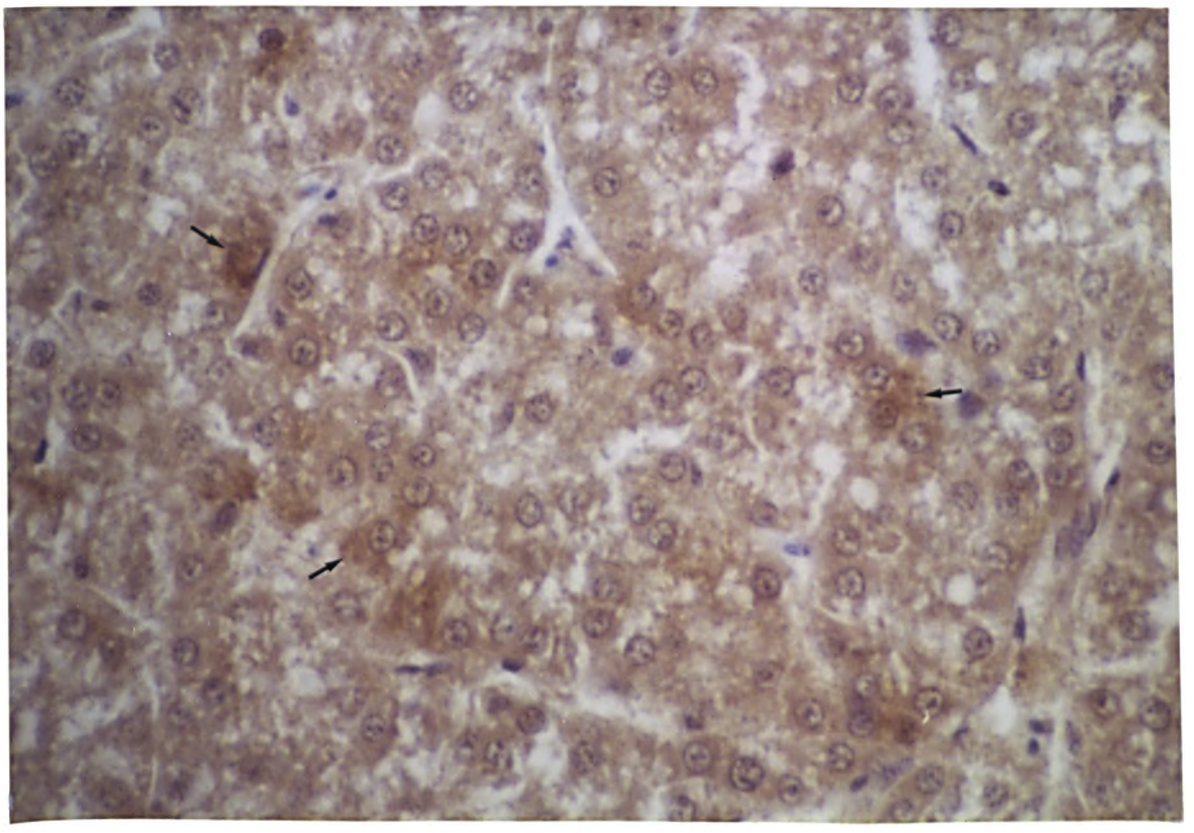


Fig. IV.6. Diffuse immunoperoxidase staining of reoviral antigen in the cytoplasm of hepatocytes (arrow) at day 2 p.i. (ABC method, DAB chromogen, haematoxylin counterstain, X677).

vesicles usually contained groups of 3 to 10 particles, although larger vesicles packed with numerous viral particles were occasionally observed (Fig. IV.8). These virus-containing vesicles were another consistent finding until the end of the experiment (96 hours p.i.), and they were also found in the epithelial cells of the bursal lining during the same period of time as in the intestine.

4. DISCUSSION

This study concentrated on the entry, initial replication and sequential spread of avian reovirus during the early stage of experimental infection in chickens. The results indicate that following oral infection in day-old chicks avian reovirus infects and multiplies in the intestine and bursa of Fabricius and subsequently spreads to most extra-intestinal organs. The widespread distribution of avian reovirus in various tissues of chickens has been reported previously by Menendez et al. (1975b), Ellis et al. (1983) and Kibenge et al. (1985).

The isolation of virus from cloacal swabs as early as 2 hours p.i. indicates that following oral administration of virus suspension in day-old chicks, the virus, presumably the inoculum, could pass very quickly through the gastro-intestinal tract.

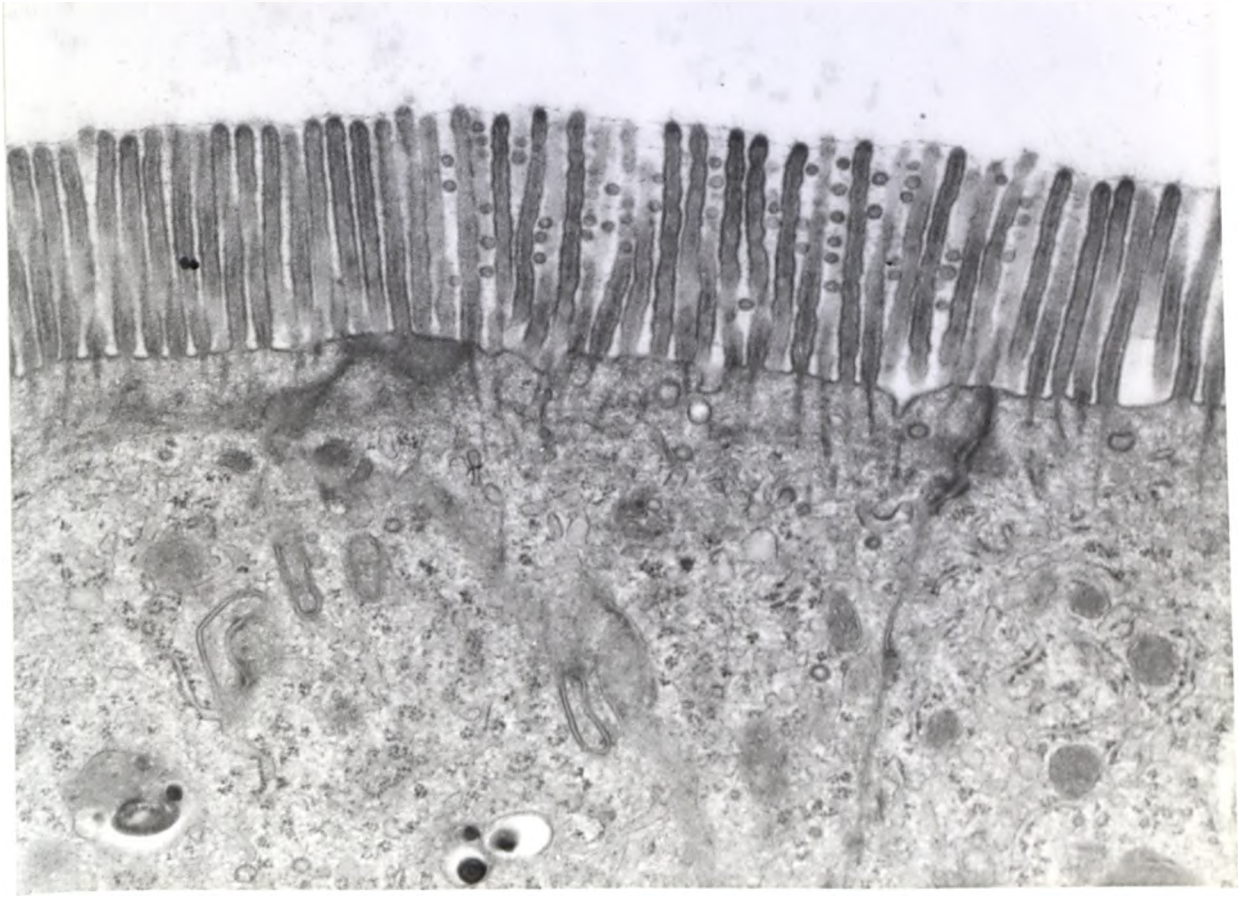


Fig. IV.7a. Electron micrograph of an ultrathin section of jejunum at 6 hours p.i. showing many viral particles between enterocyte microvilli. The microvilli of two adjacent cells are free of viral particles (X22,000).

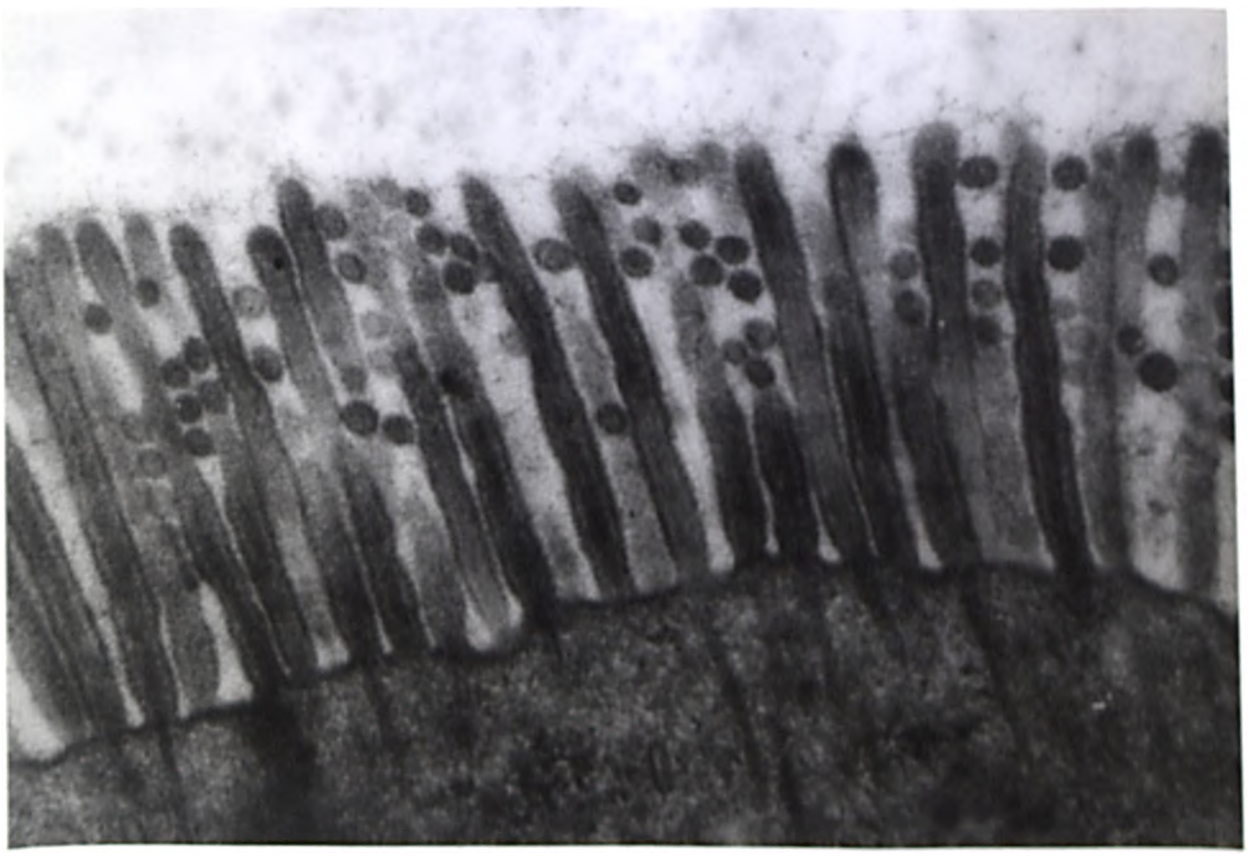


Fig. IV.7b. A higher magnification of the surface of a jejunal enterocyte at 6 hours p.i. There are numerous viral particles between the microvilli (X50,000).

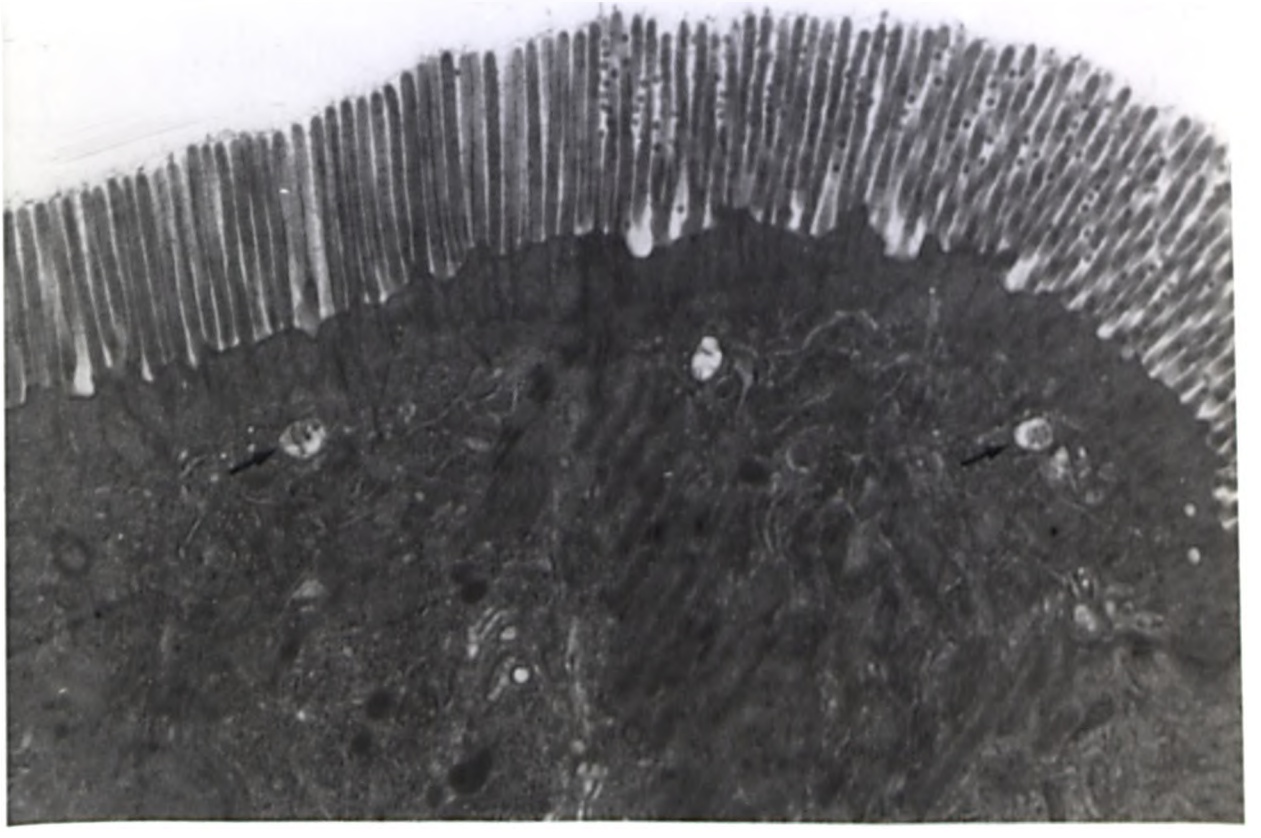


Fig. IV.8a. Electron micrograph of an ultrathin section of ileum at 24 hours p.i. Viral particles are between the microvilli of one cell and also within cytoplasmic vesicles (arrows) (X 17,000).

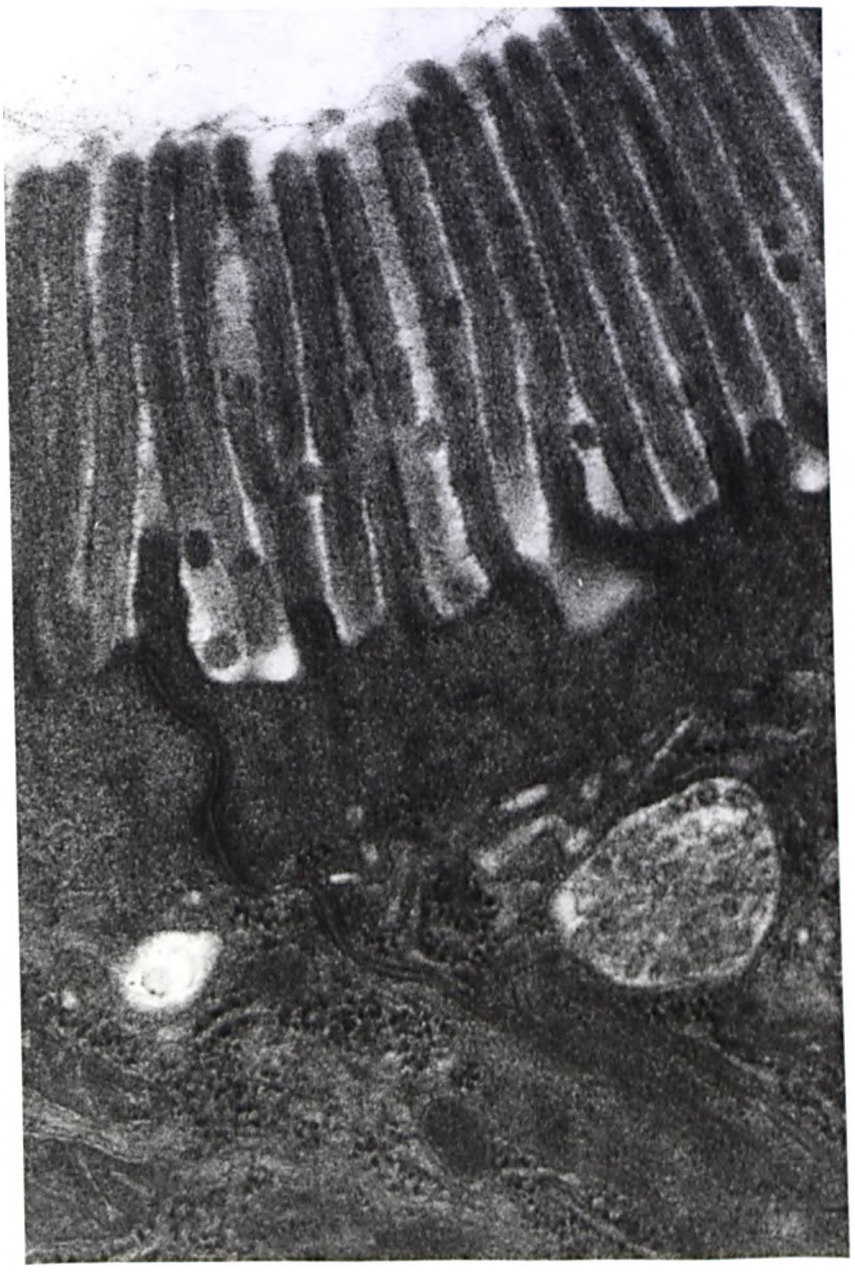


Fig.IV.8b. A portion of an epithelial cell of the ileum at 24 hours p.i showing a vesicle containing a large number of viral particles. Few viral particles are also present between the microvilli (X41,000).

Fig. IV.8c. A high magnification electron micrograph of a portion of an epithelial cell of the jejunum at 24 hours p.i. showing a vesicle containing a small number of viral particles (110,000).

It would appear from the results of virus titration, immunohistochemical staining and electron microscopy that the intestinal and bursal epithelium serve as the portal of entry and the site of primary replication of avian reovirus following oral infection. Viral particles were first seen in the vesicles of intestinal and bursal epithelium at 12 hours p.i. by electron microscopy and this would suggest that the virus enters into the epithelial cells of the intestine and bursa of Fabricius within a few hours of infection.

Following a transient drop due to disappearance of the inoculum, intestinal viral titres started to rise within 12 to 24 hours after infection. Positive FA and IP staining confirming this were evident in intestinal epithelial and sub-epithelial cells from 24 hours p.i. These results indicate that initial primary replication of avian reovirus in the intestine of chickens occurs between 12 and 24 hours p.i.

The presence of virus at a very high titre corresponding with a high level of positive FA and IP staining in the bursa of Fabricius at 24 hours p.i. would suggest that this organ may serve as a site of primary replication of avian reovirus. A similar suggestion was made by Tang and Fletcher (1987) on the basis of positive IP staining in the bursa of Fabricius.

Although the viral titres in the posterior parts of the intestine were comparatively higher than those in the anterior regions, FA and IP staining showed that

the duodenum and jejunum were the regions containing the highest proportion of virus-infected cells. It is possible that in the intestine, primary multiplication of virus took place mainly in the duodenum and jejunum, and the viral titres in the posterior part of the intestine to some extent reflected the amounts of virus shed from the duodenum and jejunum.

The FA and IP staining appeard to be useful for detecting reoviral antigen in chicken tissues. With the exception of joint tissues the background or non-specific staining in FA was negligible However, with the IP technique the background staining was a major problem probably due to the presence of non-absorbable antibodies in the primary antiserum. Although the absorption of primary antiserum with chicken liver homogenate proved to be useful in decreasing background staining in infected cell culture (Chapter III, Part 1), this was not sufficient to remove non-specific staining in sections. Trypsin treatment of sections prior to staining probably unmasked some antigenic sites as it enhanced the staining intensity and increased the number of positively stained cells. However, the tissues fixed in formol-sublimate appeared to be more suitable for trypsin treatment as compared with Bouin's-fixed tissues as the latter usually became overdigested during the treatment. Failure to detect viral antigen in the hock joints and some other tissues could be due to the weak definition of antigen localization because

of low concentration of antigen and high background staining.

Since a strong immunohistochemical staining was seen in the intestinal epithelial cells and numerous virions were observed between the microvilli of certain cells by electron microscopy throughout the experiment, it was expected that many enterocytes would contain large intracytoplasmic crystalline arrays of reovirus being assembled as described for the liver of infected chicks (Mandelli et al., 1978). It was, however, puzzling that such a stage of viral replication could not be seen in the present study, although some enterocytes contained virus in intracytoplasmic vesicles. The failure to demonstrate a sequence of events in the assembly of progeny viruses inside the cell could be due to limited sampling as only one section was examined from each organ of each bird on each occasion, and also the fact that only a proportion of cells were infected at any time. It is also possible that the assembly of progeny viruses in the surface epithelium was immediately followed by disruption of the cells and release of virus into the lumen, and the normal rapid replacement of enterocytes may have further reduced the chances of observing the later stages of viral replication. These facts could explain the presence of the large number of viral particles between the microvilli in the later stages of infection. It is likely that most of the positive immunohistochemical

staining of the intestinal and bursal epithelium represented early viral protein antigen which might have gone unrecognized as developing viral particles during electron microscopic examination.

It is interesting to note that, virus could be isolated from the liver and kidney as early as 6 hours after infection. Early isolation of virus from liver also has been reported by Kibenge et al. (1985). It is unlikely that virus reached these organs after a cycle of primary replication in the intestine or bursa in such a short period of time. An explanation may be that virus is transported directly across the surface epithelial barrier, probably via the macromolecular transport system. Following experimental infection in mice, mammalian reovirus is transported across the intestinal epithelium through specialized follicle-associated microfold (M) cells overlying the Peyer's patches (Wolf et al, 1981; 1983). Specialized follicle-associated epithelial cells or M cells, capable of macromolecular pinocytosis, have also been reported to occur in the bursa of Fabricius, caecal tonsil and ileal Peyer's patches of chickens (Bockman and Cooper, 1973; Befus et al., 1980; Burns, 1982), although the latter two organs are poorly developed in newly hatched chicks. However, the follicle-associated epithelial cells of the bursa of Fabricius of 1-day old chicks are fully capable of macromolecular uptake and could possibly transport viral particles across the epithelium

to the underlying lymphoid tissues or intercellular spaces as they do for various macromolecules (Bockman and Cooper, 1973; Sorvari, and Sorvari, 1977). Subsequently, these viral particles would be circulated to distant organs via the lymph or blood stream. Tang et al. (1987) also considered the bursa of Fabricius to be an important portal of entry for reovirus infection.

Virus was detected in serum between 2 and 5 days p.i., and rose to maximum titres ($10^{2.9}$ $TCID_{50}$/ml of serum) at days 3 and 4 p.i. However, Kibenge et al. (1985) were able to detect virus in partially washed erythrocyte fraction and plasma at 24 and 30 hours p.i., respectively. It appears that a major part of the viraemia is associated with plasma, although Kibenge et al. (1985) were able to isolate virus occasionally from the blood mononuclear cells. The exact time of onset of viraemia, however, could be even earlier than 24 hours p.i., as virus was found in the liver and kidney at 6 hours p.i. and in the heart and pancreas at 12 hours p.i.

In this study, avian reovirus appeared to be pantropic in distribution during the early stage of infection reaching all of the tissues examined at 24 or 48 hours after infection. Similar pantropic distribution of mammalian reovirus in mice during the early stage of infection has been recorded (Kundin et al., 1966).

Despite the widespread distribution during the early stage of infection, avian reoviruses are known to localize and persist in joint tissues (Olson and Kerr, 1967; Marquardt et al., 1983; Jones and Kibenge, 1984), although the possibility of viral persistence in other tissues cannot be dismissed as this has not yet been fully investigated. However, the initial virus-induced damage and/or viral persistence in the joint tissues lead to the development of pathological or immunopathological lesions, probably due to the inherent nature of the connective tissues involved.

The subsequent chapters describe the pathological and immunological aspects of reovirus infection in chickens.

Chapter V

PATHOLOGY OF EXPERIMENTAL REOVIRUS TENOSYNOVITIS IN CHICKENS WITH REFERENCE TO THE INFLUENCE OF THE ROUTE OF INFECTION

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Chapter V

PATHOLOGY OF EXPERIMENTAL REOVIRUS TENOSYNOVITIS IN CHICKENS WITH REFERENCE TO THE INFLUENCE OF THE ROUTE OF INFECTION

1. INTRODUCTION

Several factors appear to influence the development of reovirus-induced tenosynovitis in chickens. These include age at infection (Jones and Georgiou, 1984), breed (Jones and Kibenge, 1984) and strain of virus (Gouvea and Schnitzer, 1982; Jones and Guneratne, 1984). Many routes of experimental infection also have been used with varying effects (Kibenge and Wilcox, 1983).

It has been suggested that reovirus-induced tenosynovitis (viral arthritis) of chicken could be useful as a model for chronic arthritis of unknown aetiology in other species including man (Walker et al., 1977; Marquardt et al., 1983). Selection of a suitable experimental approach for quick and repeated reproduction of the disease would be very useful.

This chapter examines the pathology of experimental reovirus-induced tenosynovitis and viral persistence in the gut and hock joint following infection by four different routes, and also investigates the possibility of enhancing the disease process by direct inoculation of the virus into the hock joint or foot-pad of chickens.

2. MATERIALS AND METHODS

2.1. Experimental design

An outline of the experimental design is shown in Table V.1. Chickens were infected when 1-day old and maintained in strict isolation. Different groups were housed separately in wire-floored cages in pens within an isolation unit with filtered air ventilation. Control birds were housed in a separate pen. All birds were provided with food and water ad libitum. Birds were closely observed throughout the experiment for any clinical signs of illness.

Three birds from each infected group and two from the control group were sampled every week up to five weeks after infection, when the experiment was terminated. On each occasion, before being killed by cervical dislocation, each bird was bled by cardiac puncture and swabbed from the cloaca. However, from 2 weeks onward, all surviving birds in groups FP and A were swabbed to overcome the problem of very infrequent shedding of virus following foot-pad and particularly intra-articular infection (unpublished observation). Carcasses were examined for gross lesions, and tissues were collected aseptically from the hock joint for attempted virus isolation. Blocks of hock joints and liver (two main target organs in reovirus infection) were fixed for histological examination. Tissues were collected from both right (inoculated) and left hock

Table V.1. Outline of experimental design for infection of day-old chicks with reovirus via different routes

Group	Route of infection	Dose of infecting virus (Log_{10} $TCID_{50}$ /bird)
O (n = 15)	Oral	5.3
SC (n = 15)	Subcutaneous (at right wing web)	4.3
FP (n = 15)	Foot-pad (at plantar surface of the right foot-pad)	4.3
A (n = 15)	Articular (into the joint cavity of right hock joint)	4.3
Control (n = 10)	-	-

joints of the chickens infected by the foot-pad (group FP) or articular (group A) routes but from either joint of those infected by the oral (group O) or subcutaneous (group SC) route.

2.2. Virus isolation

Virus isolation from cloacal swabs and tissues was attempted in primary CELi cell culture monolayers grown in 24-well tissue culture plates as described in Chapter II (Section 4.3). Three subsequent passages were conducted before recording a sample as negative.

2.3. Pathology

Gross lesions were recorded during post-mortem examination. Tissues were fixed in Bouin's fluid for 12 to 18 hours and then rinsed in and transferred to 70% alcohol. Tissues were routinely processed, embedded in paraffin wax, sectioned and stained with HE as described in Chapter II (Section 7). Histological lesions included hyperplasia of synovial cells and fibroblasts, infiltration of mononuclear cells and heterophils, formation of lymphoid nodules in synovium and tendon sheaths. The lesions were given a pathological score ranging from one to three on a scale of increasing severity: 1 (slight), 2 (moderate) and 3 (severe). After giving scores for each lesion in each bird, the weekly average pathological score for each group was calculated and plotted graphically.

3. RESULTS

3.1. Clinical signs

Chicks belonging to groups O and SC were transiently depressed at 2 days p.i., but were normal by 1 week p.i. In groups A and FP, in addition to this transient depression, approximately two thirds of the chicks developed a slight swelling at the site of inoculation at 2 to 3 days p.i. and were reluctant to move. Although this transient swelling subsided in all birds between 1 and 2 weeks p.i., three of those, two from group FP and one from group A, developed twisted hock joints with both legs abducted laterally or obliquely.

No other clinical abnormality developed by 5 weeks p.i., when the experiment was terminated.

3.2. Cloacal shedding of virus

The pattern of cloacal virus shedding following infection is shown in Table V.2. Virus was isolated from all infected birds at 1 and 2 weeks p.i. After 3 weeks p.i. virus isolation became less frequent. Birds infected by the articular route (group A) stopped shedding virus earlier than those in the other groups. Only one of 9 birds in group A was shedding virus at 3 weeks p.i.

3.3. Virus persistence in hock joint

The results of virus isolation from the hock joints are shown in Table V.3. Virus was isolated from all

Table V.2. Pattern of cloacal virus shedding in chicks infected at day-old with reovirus by different routes

Route of infection	Weeks after infection				
	1	2	3	4	5
Oral	3/3*	3/3	2/3	1/3	0/3
Subcutaneous	3/3	3/3	1/3	2/3	0/3
Foot-pad	3/3	10/12	2/8	1/6	0/3
Articular	3/3	10/11	1/9	0/5	0/3
Control	0/2	0/2	0/2	0/2	0/2

* Number from which virus isolated / number examined.

Table V.3. Isolation of virus from the hock joints of chicks after infection at day-old with reovirus by different routes

Route of infection	Weeks after infection				
	1	2	3	4	5
Oral	3/3*	3/3	3/3	2/3	3/3
Subcutaneous	3/3	3/3	3/3	3/3	3/3
Foot-pad (right)	3/3R 3/3L	3/3R 3/3L	3/3R 3/3L	3/3R 3/3L	3/3R 2/3L
Articular (right)	3/3R 3/3L	3/3R 3/3L	3/3R 3/3L	3/3R 2/3L	2/3R 3/3L
Control	0/2	0/2	0/2	0/2	0/2

* Number from which virus isolated / number examined.

'R' and 'L' indicate right and left hock joints, respectively.

samples at weeks 1, 2 and 3 but less consistently at 4 and 5 weeks p.i. At that time, isolation of virus was very difficult and usually needed two or three blind passages. Two samples at four weeks p.i., one from group O and the other from group A; and two samples at 5 weeks p.i., one from group FP and the other from group A, were negative for virus isolation after 3 subsequent blind passages. In groups FP and A, virus was isolated from both left and right hock joints as early as 1 week p.i., after virus had been inoculated in the right foot-pad and right hock joint, respectively.

3.4. Gross lesions

Variations in the extent and distribution of gross lesions in hock joints in different groups are shown in Fig. V.1. The first gross lesion in hock joints was petechial haemorrhage in the synovial membrane and sheaths of digital flexor tendons. This was followed by the appearance of yellow gelatinous exudate between digital flexor tendons. One important difference in gross lesions was the presence in groups FP and A only of numerous punctate opaque foci in the articular cartilage on the distal tibiotarsus of the hock joints of the right (inoculated) legs. No cartilage change was observed in groups O and SC up to five weeks p.i., when the experiment was terminated.

At one week p.i. the edges of the livers of two birds in groups O and SC contained scattered white necrotic miliary foci.

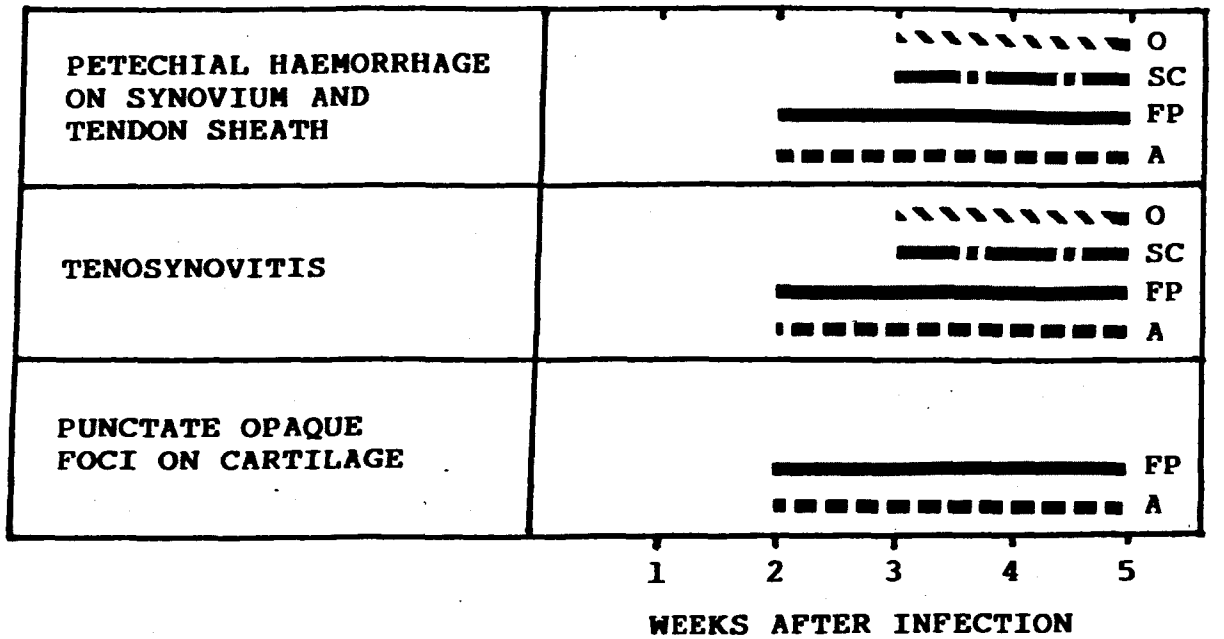

Fig. V.1. Extent and distribution of gross lesions in the hock joint of chickens after infection at day-old with reovirus by oral (O), subcutaneous (SC), foot-pad (FP) and articular (A) routes.

3.5. Microscopic lesions

The histological features of the joint lesions were similar in all groups. Pathological scores indicative of differences in the severity of lesions are shown in Fig. V.2. Microscopic lesions were more severe in parenterally infected birds, especially in those of groups FP and A, than in group O which was infected orally.

Vacuolation of the inner layer of the gastrocnemius tendon, hyperplasia of synovial cells and fibroblasts of tendon sheaths were seen as early as 1 week p.i. (Fig. V.3). Vacuolation of the tendons usually disappeared by 2 weeks p.i. leaving slight fibroplasia and disorientation of tendon collagen fibres. These changes were followed by diffuse infiltration of mononuclear cells at 2 weeks p.i. (Fig. V.4), and eventually by the formation of lymphoid follicles between tendons at about 4 weeks p.i. (Fig. V.5). Scattered infiltration of heterophils in the tendon sheaths was usually seen at about 3 weeks p.i. Lesions in the synovial membrane were essentially the same as in the tendon sheaths.

Lesions in the articular cartilage of the distal tibiotarsus were seen between 2 to 5 weeks p.i., but only in the right (inoculated) joints of birds infected via the foot-pad or intra-articular route. At 2 weeks p.i., they appeared as randomly distributed superficial focal degeneration and necrosis, characterized by pyknosis and karyorrhexis of the nuclei of chondrocytes on

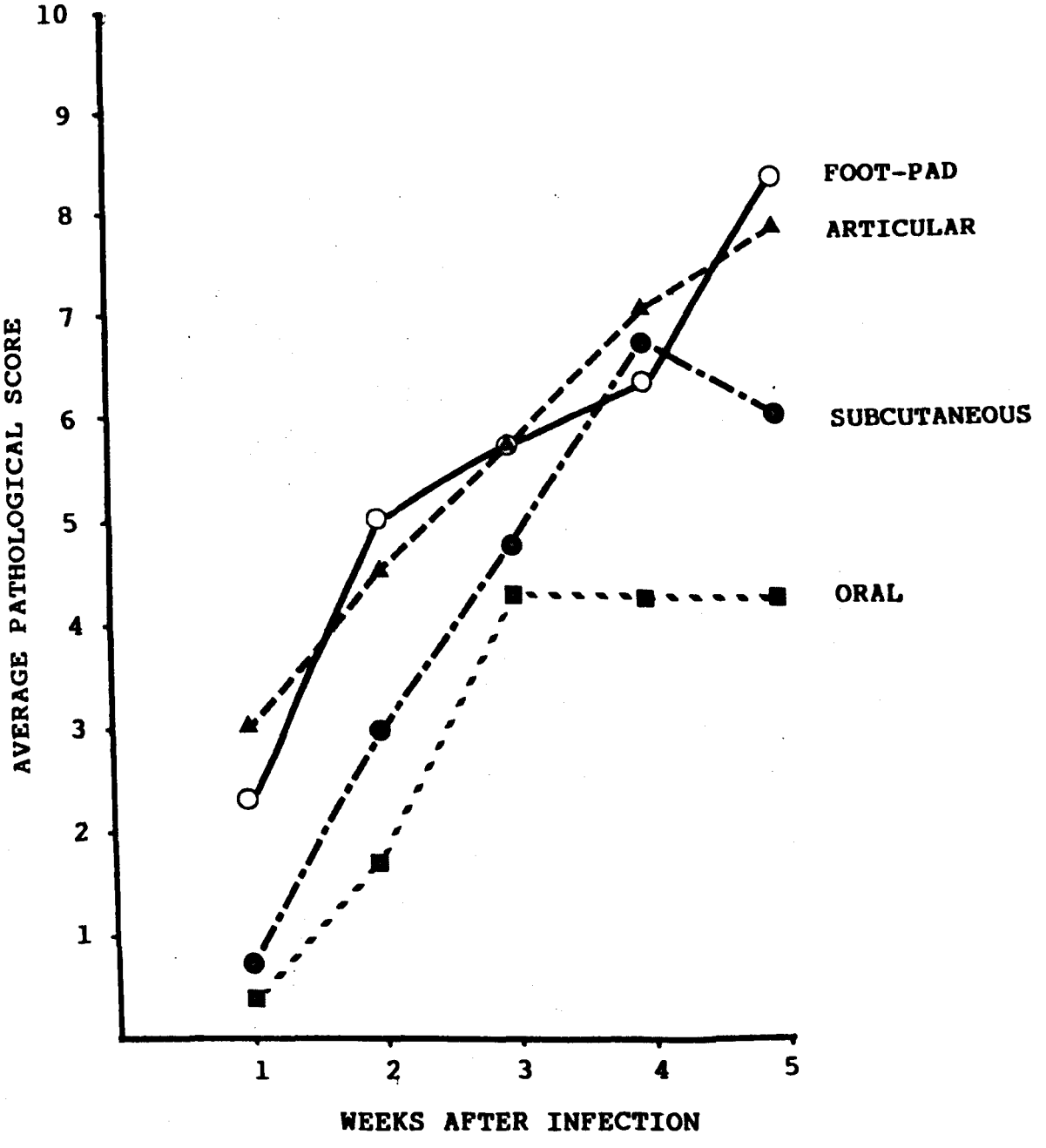

Fig. V.2. Relative severity of microscopic lesions in the hypotarsus of chickens after infection with reovirus at day-old by different routes (see Section 2.3 for scoring system for lesions).

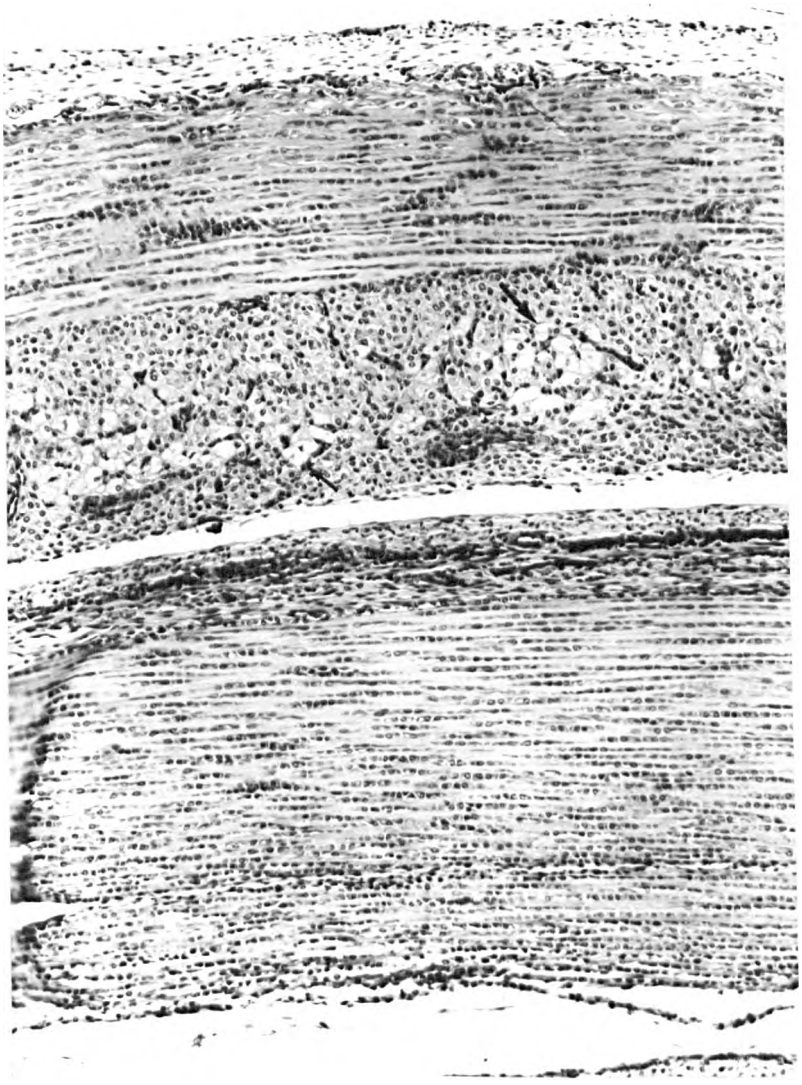

Fig.V.3. Hypotarsus 1 week after infection. Vacuolations in the inner layer of gastrocnemius tendon (arrow) (HE, X162).

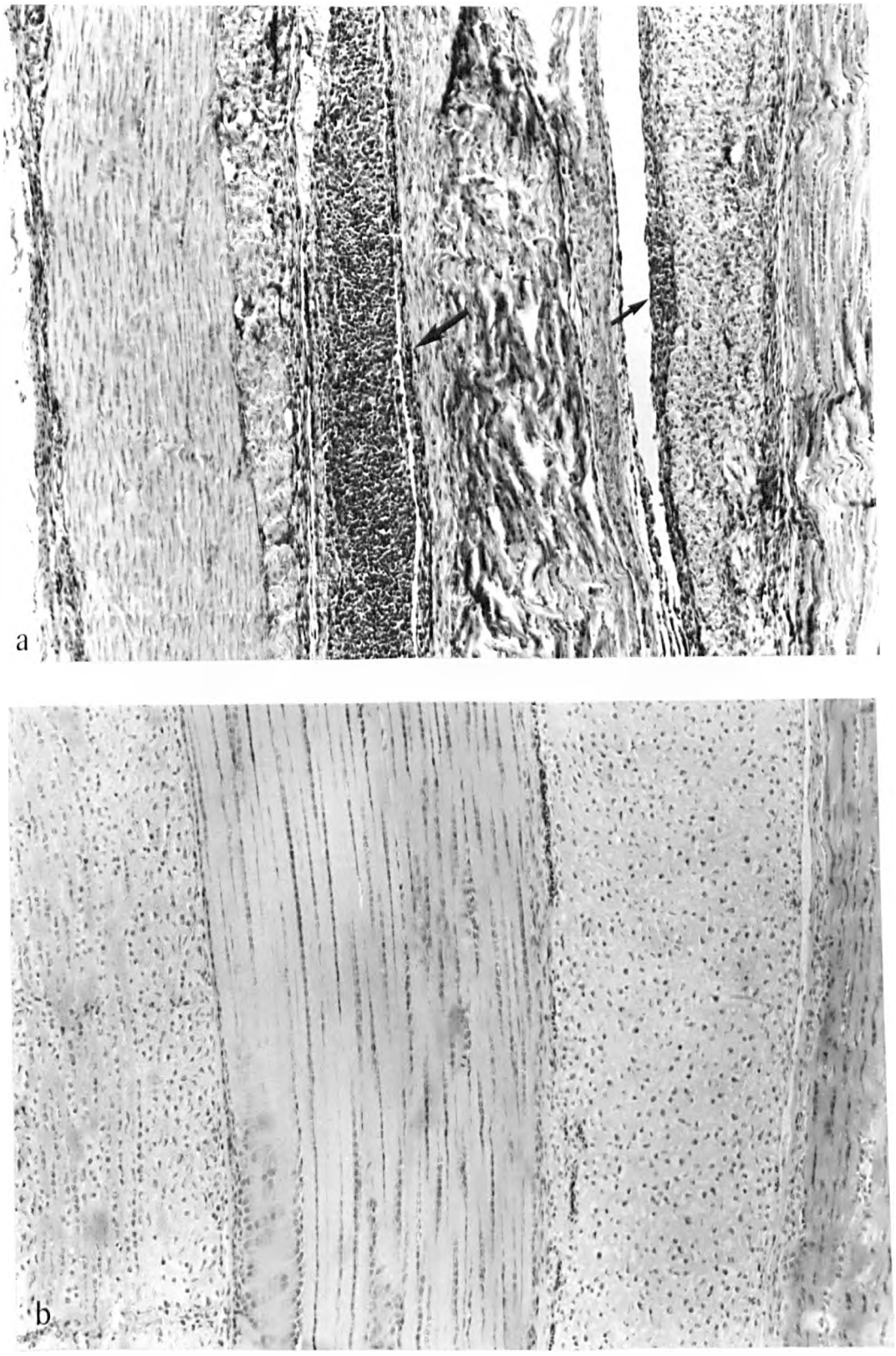

Fig.V.4a. Hypotarsus 2 weeks after infection. Diffuse accumulation of mononuclear cells in tendon sheaths (arrow) (HE, X162).

b. Hypotarsus of age-matched uninfected control chicken (HE, X120).

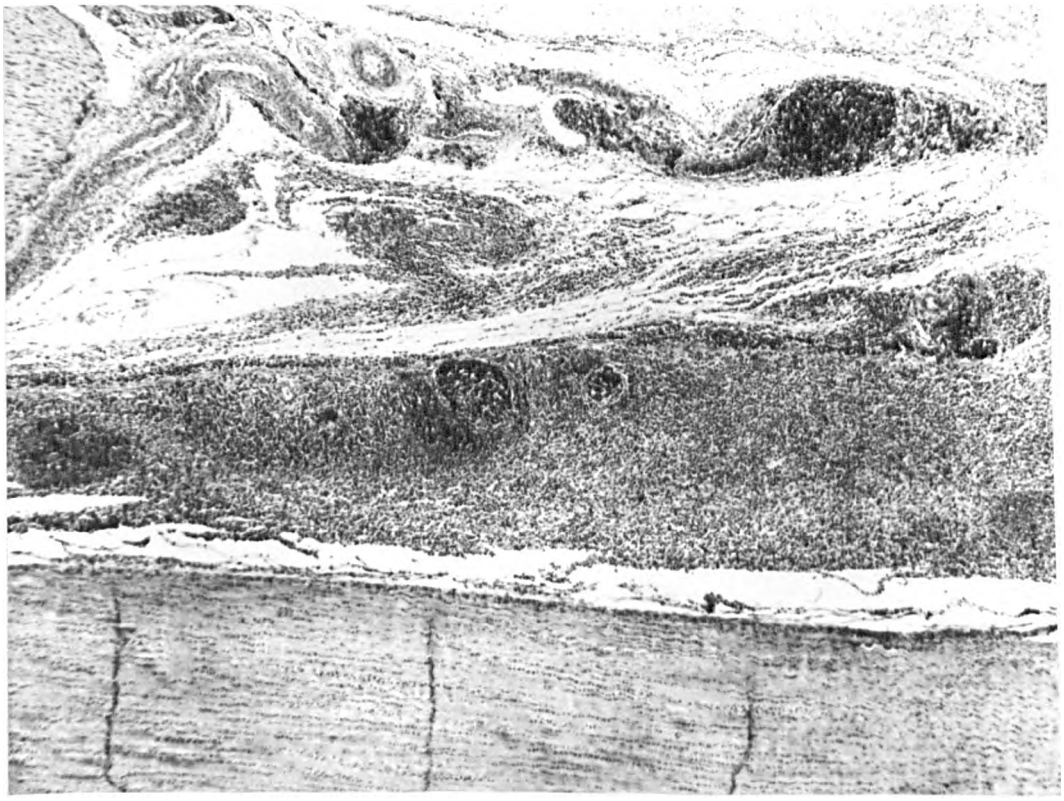

Fig.V.5. Hypotarsus 4 weeks after infection. Diffuse and follicular aggregations of mononuclear cells in tendon sheaths (HE, X65).

the articular surface (Fig. V.6). This was followed by focal partial dissolution of cartilage matrix at 3 weeks p.i. (Fig. V.7), which became more extensive at 4 and 5 weeks p.i. (Fig. V.8).

At 1 week p.i. the liver contained hydropic, vacuolated, and few scattered pyknotic hepatocytes (Fig. V.9). Livers of 2 birds with gross hepatic lesions contained subcapsular eosinophilic necrotic masses surrounded by fibrous tissues and a few giant cells. Infiltration of mononuclear cells and heterophils started at 2 and 3 weeks p.i., respectively.

4. DISCUSSION

Although no severe clinical disease was produced in these light breed chickens during this short observation period, the typical lesions of tenosynovitis (Kerr and Olson, 1969; van der Heide et al, 1974; and Jones and Onunkwo, 1978) developed following infection by all four routes. As sham inoculation was not done in control chicks, it cannot be assumed that the transient local swelling at the site of inoculation and reluctance to move following foot-pad and intra-articular injection was due solely to the inflammation elicited by the infection.

Microscopic lesions in the joint were more severe in birds which had been infected through the foot-pad

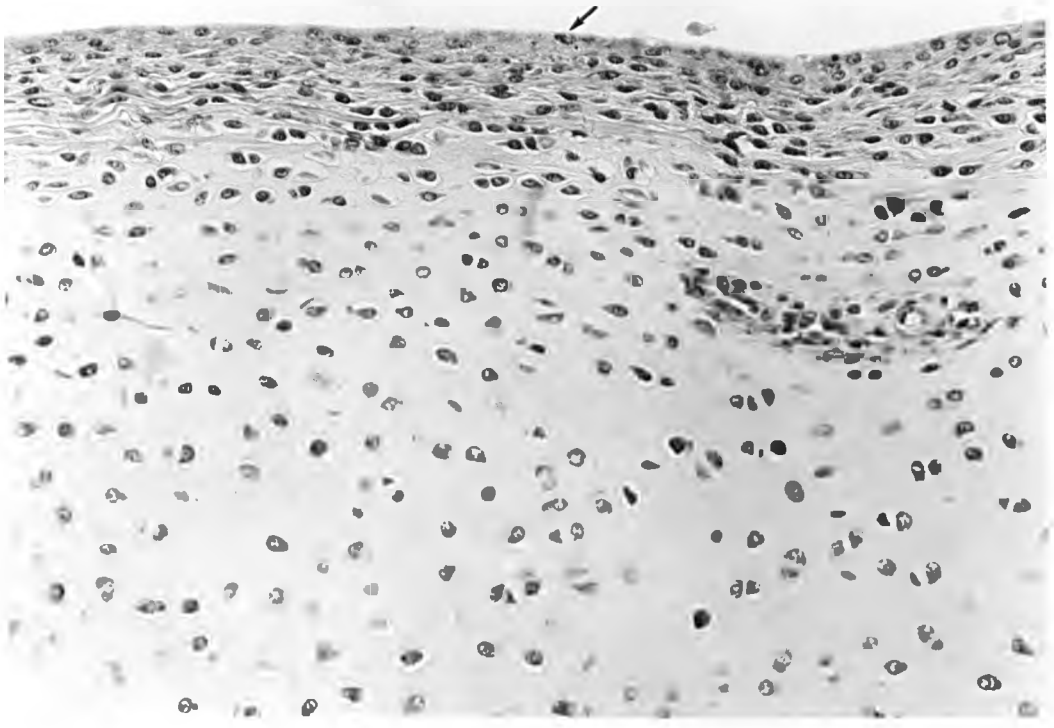

Fig.V.6. Distal tibial cartilage 2 weeks after infection. Pyknosis and karyorrhexis (arrow) in the nuclei of chondrocytes of articular surface (HE, X300).

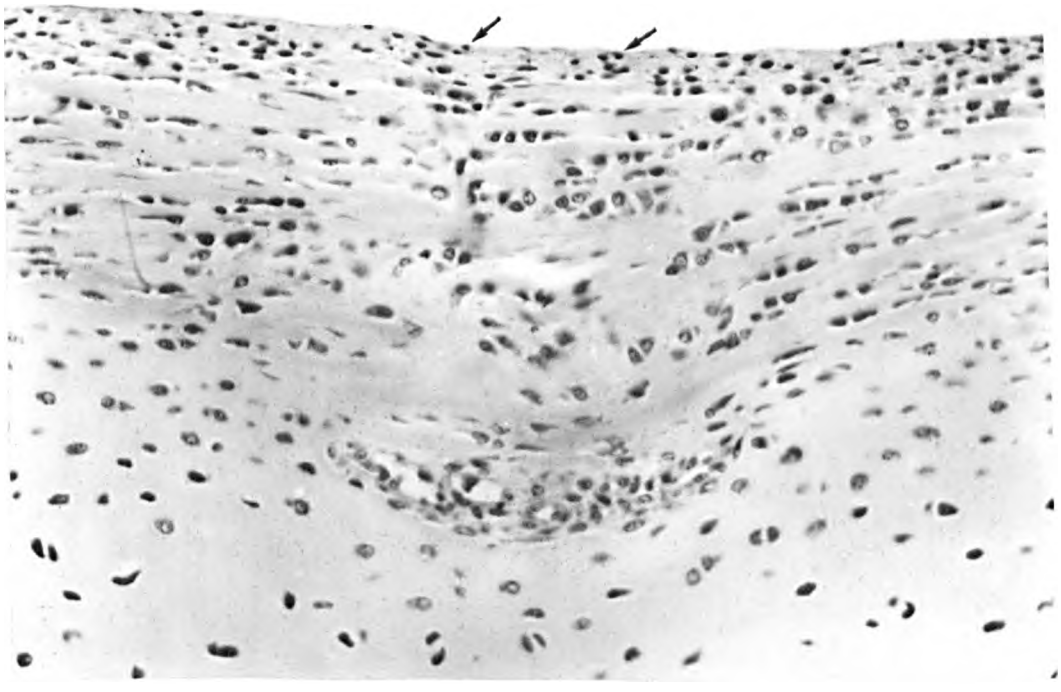

Fig.V.7. Distal tibial cartilage 3 weeks after infection. Pyknosis of chondrocyte nuclei (arrow) and partial dissolution of cartilage matrix (HE, X300).

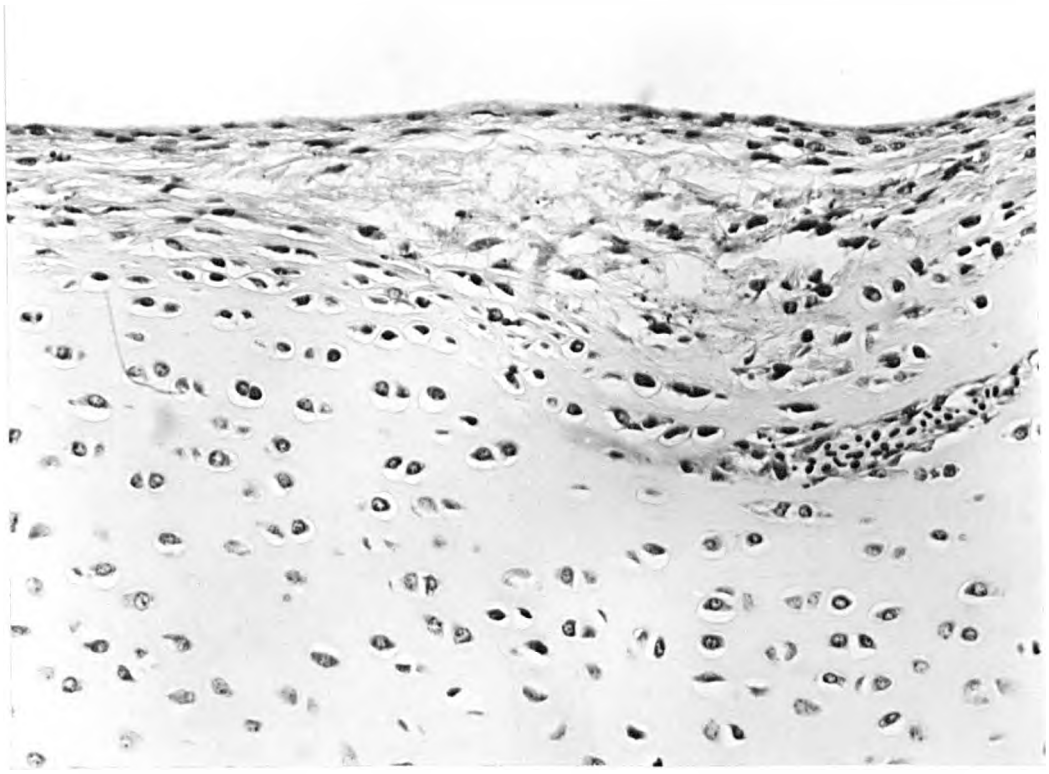

Fig.V.8. Distal tibial cartilage 4 weeks after infection. Necrosis of chondrocytes and extensive dissolution of cartilage matrix (HE, X300).

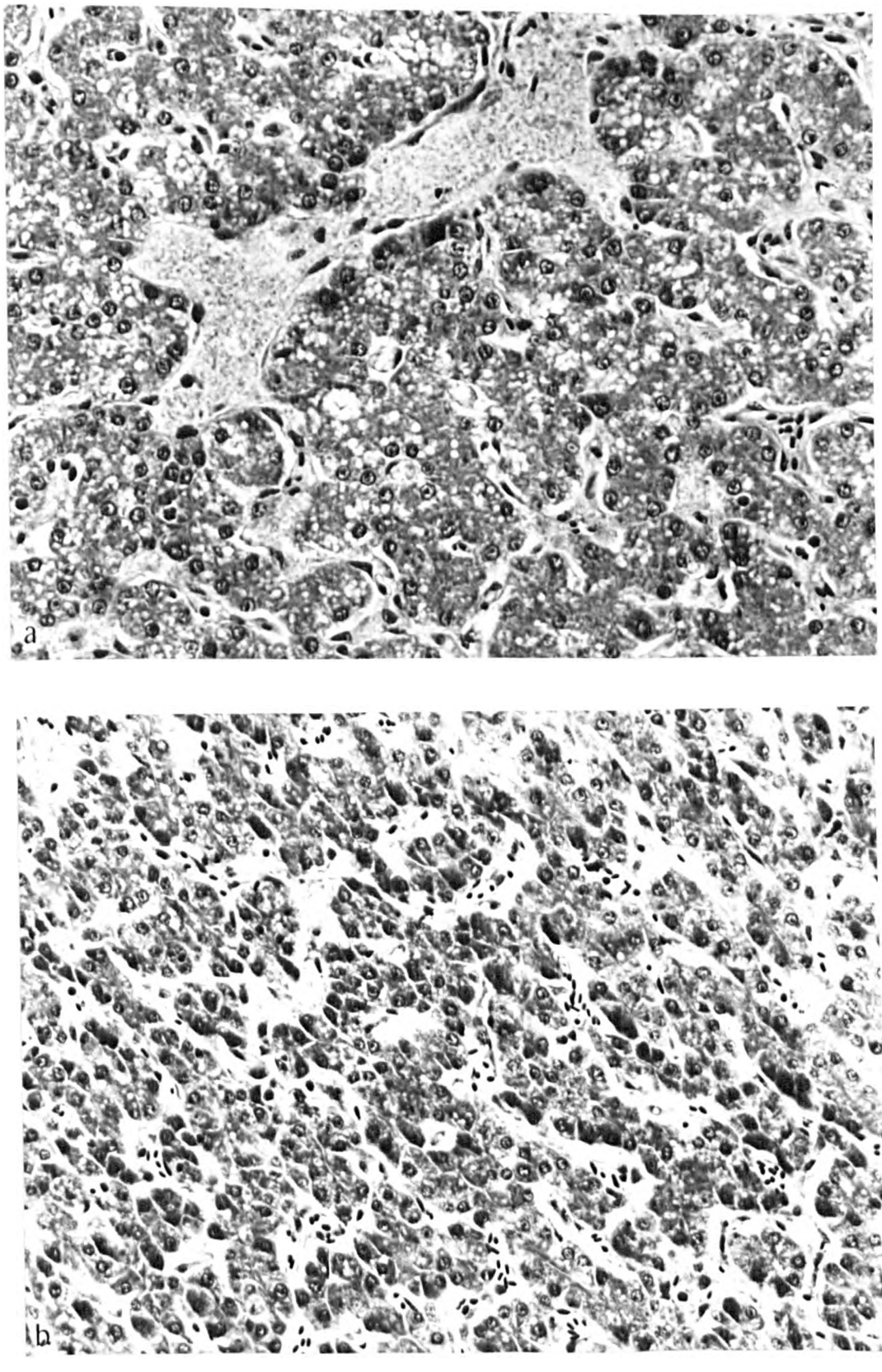

Fig.V.9a. Liver 1 week after infection. Showing vacuolation of hepatocyte cytoplasm (HE, X455).

b. Liver of age-matched uninfected control chicken (HE, X300).

or intra-articular route than in those infected orally or subcutaneously. Wood and Thornton (1981) also observed a greater pathological response in chickens following foot-pad infection when compared with oral infection.

It is interesting that cartilage lesions appeared as early as 2 to 3 weeks p.i. in the hock joints of birds infected through foot-pad or intra-articular route. That such lesions were seen only in these 2 groups during this experimental period may be explained as part of the more severe disease process resulting from direct inoculation of virus into the joint or its close vicinity. It is likely that these changes could have led to cartilage erosion at a later stage of infection such as observed by Kerr and Olson (1969) and Jones and Kibenge (1984) at 43 days and 12 weeks p.i., respectively.

Focal degenerative and necrotic lesions of articular cartilage might be due to the direct effect of virus infection, or the action of inflammatory mediators or proteolytic enzymes released from tissues or serum during the disease process. Enzymes such as plasmin, or monokines such as interleukin 1, may damage articular cartilage (Lack and Rogers,1958; Krakauer et al., 1985).

Lesions in the liver of all groups were essentially the same as those previously described by Mandelli et al. (1978). The infiltration of lymphocytes following recovery from early degenerative and necrotising changes in liver would be difficult to evaluate

in individual birds as a few small lymphoid foci may be found in normal avian parenchymatous tissues (King and McLelland, 1984), but the greater frequency and size of the lymphoid foci in infected birds were clear when the liver sections were compared with those of normal control birds.

Isolation of virus as early as 1 week p.i. from cloacal swabs in all groups suggests that enteric infection occurred following inoculation of virus by any route. However, birds infected through the articular route stopped shedding virus earlier than those in other groups. This might be due to the fact that direct inoculation of virus into the joint partially reduced the opportunity for the spread of virus from articular cavities whose vascular supply is confined to the synovial connective tissues.

The result of attempts to isolate virus from hock joints supports the concept of persistent infection. Persistence of virus can occur in joints for as long as 13 weeks (Jones and Onunkwo, 1978), 115 days (Marquardt et al., 1983) or 285 days p.i. (Kerr and Olson, 1969). The relative difficulty and the occasional failure to isolate virus at later stages from experimentally and naturally infected clinical cases (Jones et al, 1981) might be related to the strategic adaptation of virus to the establishment of persistent infection. Studies with mammalian reovirus indicated that mutation of viral genes was associated with the

establishment of persistent infection, which ultimately resulted in the production of defective virus particles with altered temperature sensitivity, and low yield or small plaques (Sharpe and Fields, 1985). An association between viral persistence and the mutation of viral genes also has been reported recently for avian reovirus (Haung et al., 1987).

Although the isolation of virus from joints was difficult and less frequent in the later stages of infection, the lesions were still progressive. This raises the possibility that the lesions of tenosynovitis might be, in part at least, an autoimmune consequence of virus infection. Autoimmunity to pancreas, pituitary gland and gastric mucosa has been reported following mammalian reovirus infection in mice (Haspel et al., 1983). Moreover, it is possible that the pathogenesis of avian viral arthritis might share some of the autoimmune mechanisms involved in the pathogenesis of different connective tissue disorders such as human rheumatoid arthritis, (Holborow and Swannell, 1977).

The next chapter describes the immune responses of chickens to experimental reovirus infection.

Chapter VI

SOME ASPECTS OF THE IMMUNOLOGY OF AVIAN REOVIRUS INFECTION

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Chapter VI

SOME ASPECTS OF THE IMMUNOLOGY OF AVIAN REOVIRUS INFECTION

The outcome of a microbial infection depends in part on host immune responses. In a favourable situation, host defence mechanisms effectively eliminate the invading microorganism and the host recovers from the infection. However, under different circumstances the microorganism may partially or totally overcome host defence and either causes death or establishes a persistent infection. Detailed investigation of the immunology of any infection is necessary to fully understand the pathogenesis and this chapter deals with a number of aspects of the immunology of experimental reovirus infection in chickens. The first part of this chapter presents the results of some studies on the humoral and cellular immune responses of chicks to experimental reovirus infection; the second part elucidates the role of maternal antibodies in protection; and the final part investigates the possible involvement of autoimmune mechanisms in the pathogenesis of the infection.

Part 1.

IMMUNE RESPONSES OF CHICKENS TO EXPERIMENTAL REOVIRUS INFECTION

1. INTRODUCTION

For diagnostic purposes, humoral antibody to avian reovirus infection has most commonly been detected using AGP tests. VN methods have been used for serological comparision of avian reoviruses. Little has been reported on the sequential humoral antibody responses by these methods, and there are no reports of immunological studies using ELISA. Two communications in which VN has been used for this purpose are those by Mukiibi-Muka et al. (1984), who compared neutralizing antibody responses of chickens to reovirus following inoculation by different routes at 3 weeks of age, and Jones and Kibenge (1984), who examined serological responses of different breeds of chickens using VN tests. The development of an ELISA method for detecting reovirus antibody has been reported by Slaght et al. (1978), but there is no report describing the use of ELISA for measuring temporal antibody responses of chickens following infection with reovirus.

Cellular immune responses are also very important in viral infections. However, nothing is known about

the cell-mediated immune responses of chickens to reovirus infection.

The present part of this chapter describes some studies on the temporal antibody responses of young chicks to experimental reovirus infection, and also presents some preliminary observations on cell-mediated immune responses of chickens following reovirus infection.

2. MATERIALS AND METHODS

2.1. Experimental design

2.1.1. Experiment 1: AGP and VN

This part of the study made use of the sera collected from a previous experiment (Chapter V), in which 1-day old chicks were inoculated with reovirus by oral, subcutaneous, foot-pad or intra-articular route. Details of the inoculation protocol and sampling schedule are given in Chapter V. Serum samples obtained at 5 weekly intervals from 4 infected and 1 control groups were examined for humoral antibody responses by AGP and VN tests. The AGP test was done on individual sera, but VN was performed with weekly pooled serum samples.

2.1.2. Experiment 2: ELISA and delayed hypersensitivity

Fifty 1-day old chicks were divided into two equal groups and housed in two separate pens. One group was

infected orally with 0.3 ml(10^5 $TCID_{50}$)/bird of the reovirus suspension prepared from infected CELi cell culture. The other group was sham-inoculated with the same amounts of uninfected CELi cell culture fluid prepared in the same way as the virus suspension. Two to four chicks from each group were sampled on days 4 and 7 p.i. and then at weekly intervals until 6 weeks p.i. Birds were bled by cardiac puncture before being killed by cervical dislocation. Apart from these, between 2 and 5 weeks p.i., a total of 6 to 10 birds from each group were exsanguinated by cardiac puncture, before being killed as above, to obtain heparinized blood for the standardization of a lymphocyte transformation assay (LTA) as described below. At 6 weeks p.i., 4 cockerels from each group were used for measuring delayed-type hypersensitivity (DTH) reaction to the virus (see below). Serum samples obtained in this experiment were analysed by an ELISA method for the humoral antibody response to reovirus. These sera were also used to investigate several other parameters related to possible autoimmune reactions involved in reovirus infection, this will be described in Part 3 of this chapter.

2.1.3. Experiment 3: LTA

Two groups of one-day old chicks, each consisting of 10 birds were infected or sham-infected essentially as described in Experiment 2. Two chicks were sampled

for LTA on each of the following occasions: weeks 1, 2, 3 and 5 p.i. About 2 to 5 ml blood was collected from each bird by cardiac puncture into a syringe containing heparin (50 to 100 iu/ml of blood) before the bird was killed by cervical dislocation. The blood was transferred to a siliconized universal bottle kept on ice and processed immediately as described below (Section 2.4).

2.2. Serological tests

The procedures for AGP and VN tests together with the basic ELISA protocol are described in detail in Chapter II (Section 5). The method of obtaining ELISA titre from corrected absorbance values are given in Chapter III (Part 2).

2.3. Delayed-type hypersensitivity test

The wattle test (Dharsana and Spradbrow, 1985) was used to examine the DTH reaction to viral antigen in reovirus-infected chickens.

2.3.1. Viral antigen

The reovirus antigen was prepared as described for the ELISA antigen (Chapter II, Section 5.3.1), but this was used after inactivation by UV light as described for mammalian reovirus (Greene and Weiner, 1980). Briefly, caesium chloride density gradient-purified virus was diluted in PBS-A to contain 1 mg protein

per ml. The diluted antigen was placed in a small petridish and exposed to a 15W UV light source of unknown wavelength in an inoculation cabinet for 10 minutes at a distance of 10 cm. After inactivation, no infectivity could be detected by the standard virus isolation technique (Chapter II, Section 4.2).

2.3.2. The test

Fifty microlitres of the viral antigen were inoculated into the right wattle of 4 infected and 4 sham-infected (Experiment 2) 6-week old cockerels which were identified by wing tags. The left wattle of each bird received 50 µl of PBS-A as control. The wattle thickness was measured with slide calipers before and 24 hours after the challenge injections.

2.4. Lymphocyte transformation assay

A recently developed colorimetric method of LTA (Mosmann, 1983; Rai-el-Balhaa et al., 1985) was used here to study the peripheral blood lymphocyte responses of reovirus-infected and uninfected chickens to a non-specific mitogen phytohaemagglutinin (PHA)* and the reovirus antigen. The method of preparation of the purified viral antigen was essentially the same as described for the ELISA antigen (Chapter II, Section 5.3.1).

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* Sigma Chemical Co. Ltd., Poole, Dorset, England.

2.4.1. Preparation of peripheral blood lymphocytes

The lymphocyte-rich fraction of peripheral blood was prepared as described by Hudson and Hay (1980). Blood was transferred to a siliconized test tube and centrifuged in a refrigerated bench centrifuge for 3 minutes at 150g followed by 10 minutes at 35g for sedimenting erythrocytes. The supernatant contained mostly leucocytes. If a loose buffy coat formed, it was resuspended by gentle stirring of the plasma with a pasteur pipette. The leucocytes were pelleted from the plasma and washed twice in Hank's balanced salt solution* by centrifugation at 150g for 10 minutes each time. The cells were resuspended in 0.5 ml of RPMI 1640 medium*, supplemented with 200mM L-glutamine, 0.01M sodium bicarbonate, 0.014M HEPES, penicillin (100 iu/ml), streptomycin (100 µg/ml), fungizone (2 µg/ml) and 5% heat-inactivated foetal calf serum. After counting the number of viable leucocytes, more medium was added to adjust the cell concentration to $1X10^7$ leucocytes per ml.

2.4.2. The assay

The cell suspension was dispensed in a flat-bottomed 96-well microtitre plate at a volume of 50 µl per well. Then equal volumes of an appropriate dilution of PHA or viral antigen in medium (see below) were added to the wells. Each dilution of the mitogen or

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* Flow Laboratories, Rickmansworth, Herts, England.

viral antigen was added in triplicate wells. One set of control wells received 50 µl cells and 50 µl medium per well, while another set of wells, used as blanks, received 100 µl medium per well only. The plate was incubated for 72 hours at 37°C in a humidified incubator containing 5% CO_2 in air. Then 10 µl of a 0.5% solution of tetrazolium salt MTT (Appendix-A) was added to each well and incubated for a further 4 hours at 37°C. The dehydrogenase enzymes of activated lymphocytes cleaved pale-yellow MTT into a dark-blue formazan product. At the end of the 4-hour incubation, the formazan crystals were dissolved by adding 100 µl acid-isopropanol (0.04N HCl in isopropanol) per well followed by vigorous pipetting. The plate was read at a wavelength of 492 nm in a microelisa reader. The results are presented as either absorbance values or stimulation ratios (absorbance of well containing cells plus mitogen or antigen / absorbance of well containing cells without mitogen or antigen).

For the initial trials, PHA was used at various dilutions in medium RPMI 1640 to obtain final concentrations ranging from 5 µg to 80 µg per ml. For Experiment 3, a final concentration of 20 µg PHA per ml was selected. The viral antigen was used in three dilutions to obtain final concentrations of 10, 2.5 and 0.625 µg protein per ml.

3. RESULTS

3.1. Humoral antibody responses of chicks to reovirus

The results of serum AGP and VN tests on chicks after infection with reovirus by different routes are shown in Table VI.1 and Fig. VI.1, respectively. ELISA antibody responses of chicks to reovirus after oral infection (Experiment 2) are presented in Fig. VI.2.

The results of all three tests indicate that following infection with reovirus at 1-day of age young chicks may take 2-3 weeks to seroconvert. In experiment 1, only one chick, which had been infected by the foot-pad route, showed a positive AGP reaction at 2 weeks p.i. However, most of the infected birds sampled at 3 weeks p.i. and all at 4 and 5 weeks p.i. were positive by the AGP test. A neutralizing antibody titre ≥ 3 $\log_2$, a level which may be considered to be specific, was first observed at 2 weeks p.i. in the sera collected from birds infected by the foot-pad and subcutaneous routes. All of the infected birds sampled at 3 weeks p.i. had neutralizing antibodies, although the titres in parenterally infected birds were four-fold higher than those in the orally infected birds.

3.2. Delayed hypersensitivity response

The result of DTH tests at 6 weeks p.i. are presented in Fig. VI.3. All four infected birds

Table VI.1. Results of agar gel precipitation test in sera from chickens infected with reovirus by different routes at 1-day of age

Route of infection	Weeks after infection				
	1	2	3	4	5
Oral	0/3*	0/3	2/3	3/3	3/3
Subcutaneous	0/3	0/2	1/3	3/3	3/3
Foot-pad	0/3	1/3	1/2	3/3	3/3
Articular	0/2	0/3	2/3	2/2	3/3
Control	0/2	0/2	0/2	0/2	0/2

* No. of serum samples having precipitin antibody / No. examined

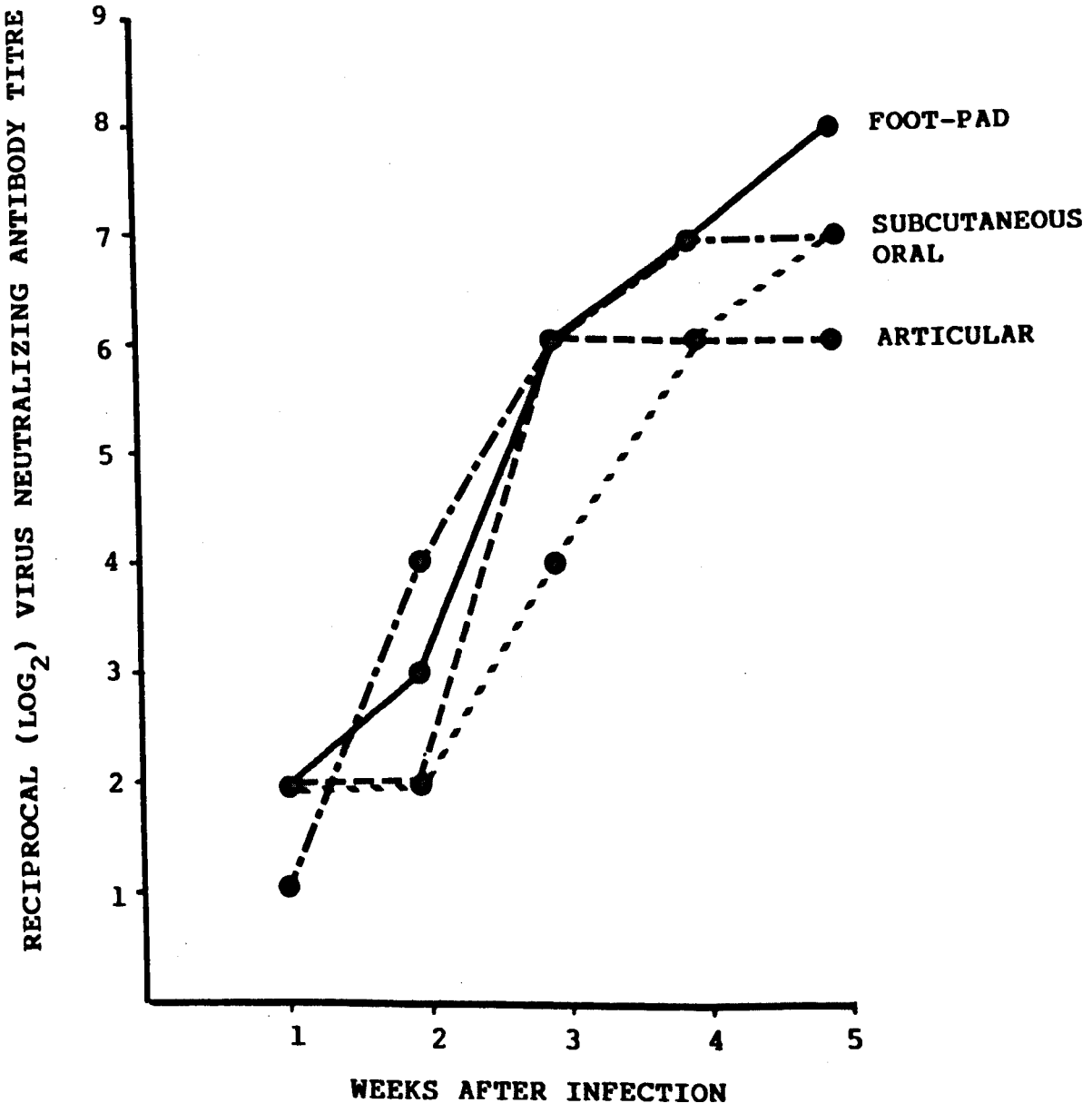

Fig. VI.1. Neutralizing antibody responses of chicks following infection with reovirus by different routes at 1-day of age. Each point represents the titre of a pool of 3 samples.

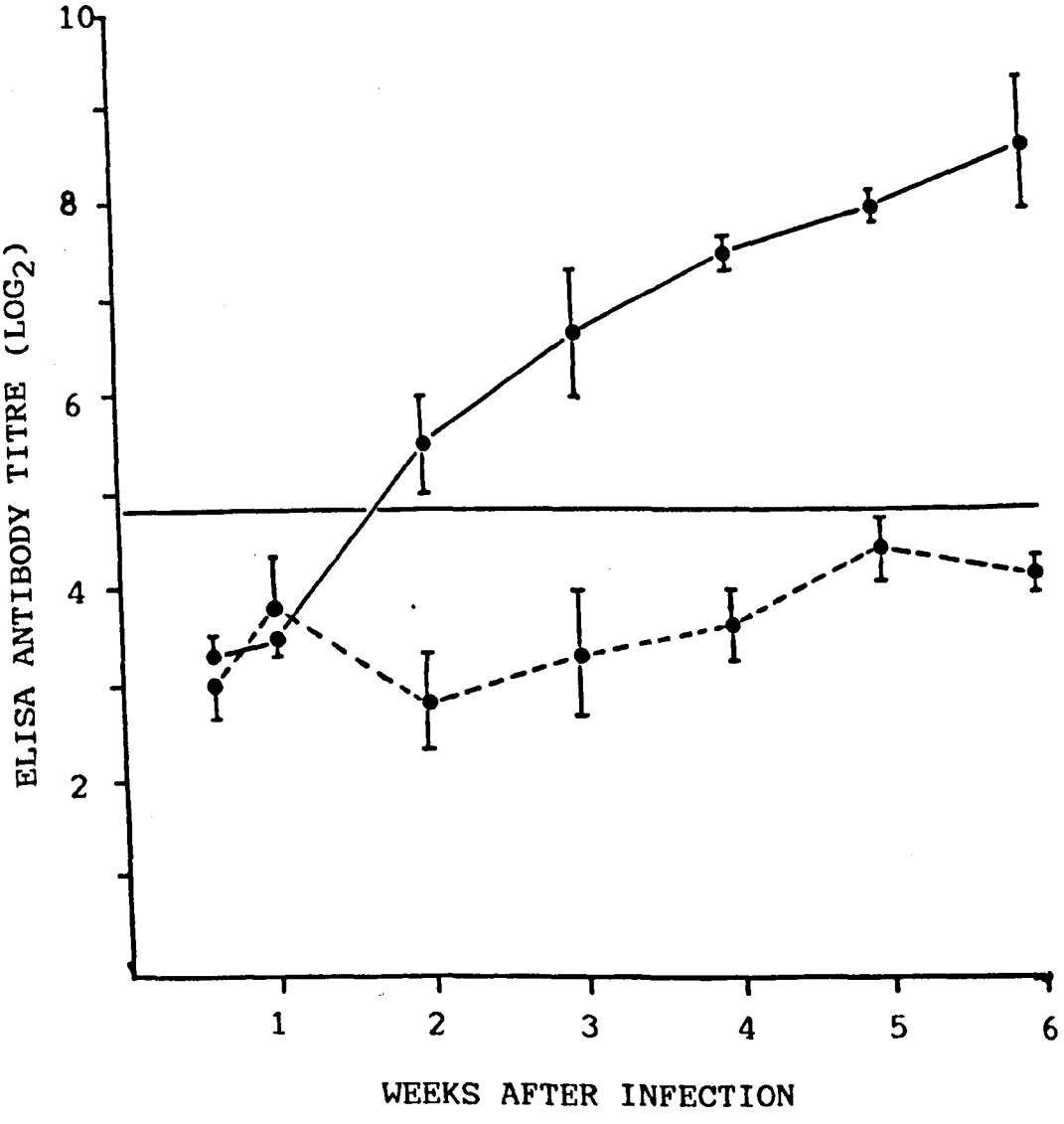

Fig. VI.2. Humoral antibody responses of chicks to reovirus as measured by ELISA after oral infection (solid line) or sham-infection (broken line) at 1-day of age. Vertical line at each point represents standard deviation of 2-4 replicate samples. Horizontal line represents positive-negative threshold baseline.

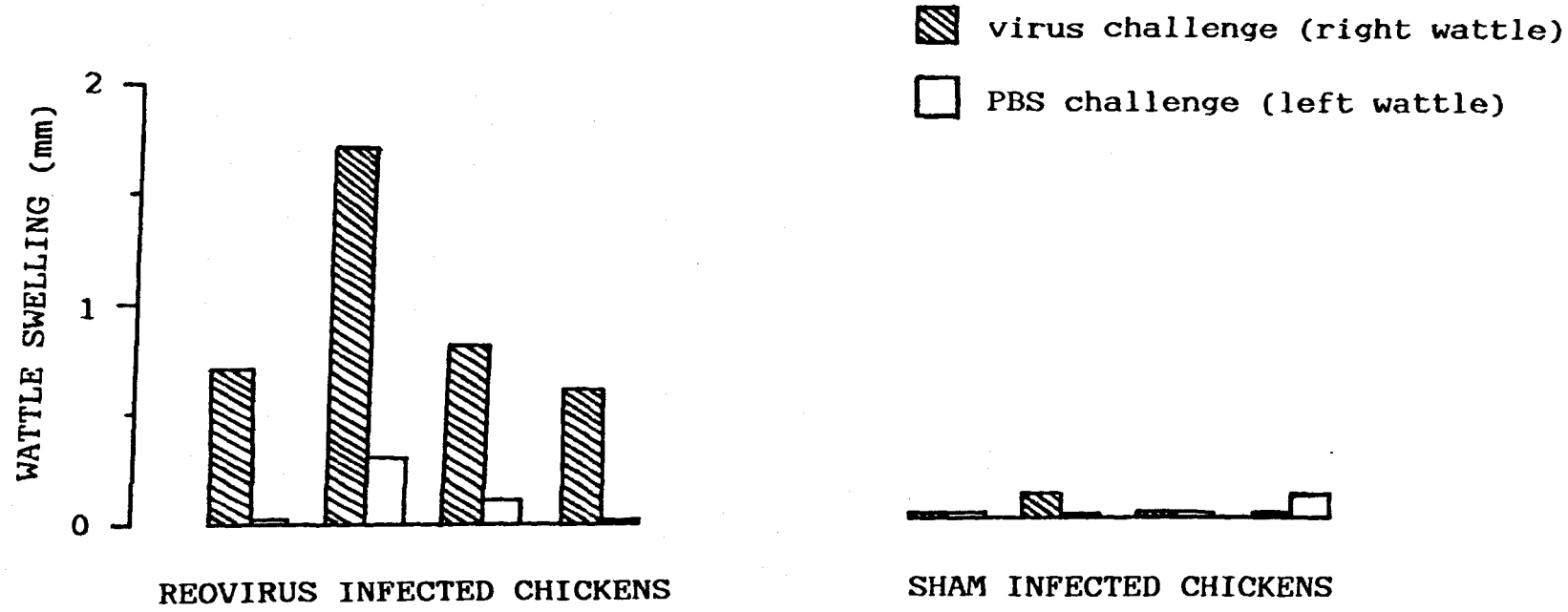

Fig. VI.3. Delayed hypersensitivity responses of chickens as measured by wattle swelling following oral infection with reovirus or sham-infection at 1-day of age and challenge with viral antigen or PBS 6 weeks later.

developed wattle swelling by 24 hours after challenge with the viral antigen, the swelling being within a range of 0.6 mm to 1.7 mm (43% to 130% greater than that of prechallenge thickness). The wattle swellings in the control group following virus challenge or in both groups following PBS challenge were negligible, the maximum being 0.3 mm.

3.3. Lymphocyte responses

Peripheral blood lymphocyte responses of three 3-weeks old control chickens (Experiment 2) to various concentrations of PHA are shown in Fig. VI.4. The stimulation of lymphocytes with PHA was indicated by increased cleavage of MTT into formazan. A maximum 3-fold increase in formazan production was observed with PHA at a final concentration of 20 μg/ml in the culture.

The responses of peripheral blood lymphocytes of reovirus-infected and sham-infected chickens to PHA are shown in Fig. VI.5. The stimulation ratios were within a range of 2 to 5. There was no significant difference ($P < 0.05$) between the lymphocyte responses of infected and control chickens when the stimulation ratios of all infected chickens were compared with those of all control chickens by Wilcoxon rank sum test. Weekly results were not compared statistically because of the limited numbers of samplings.

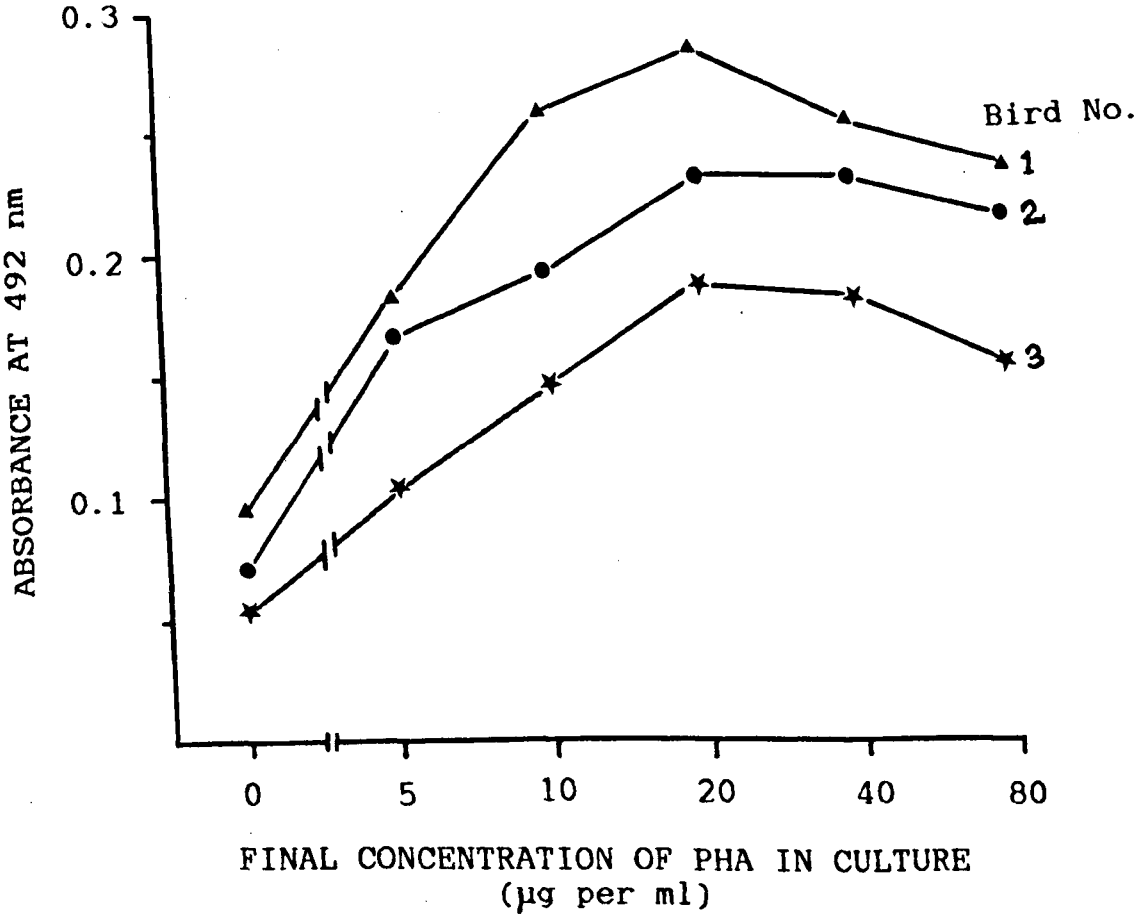

Fig. VI.4. Responses of peripheral blood lymphocytes of three 3-weeks old SPF chickens to various concentrations of phytohaemagglutinin in a colorimetric lymphocyte transformation assay.

KEY ?

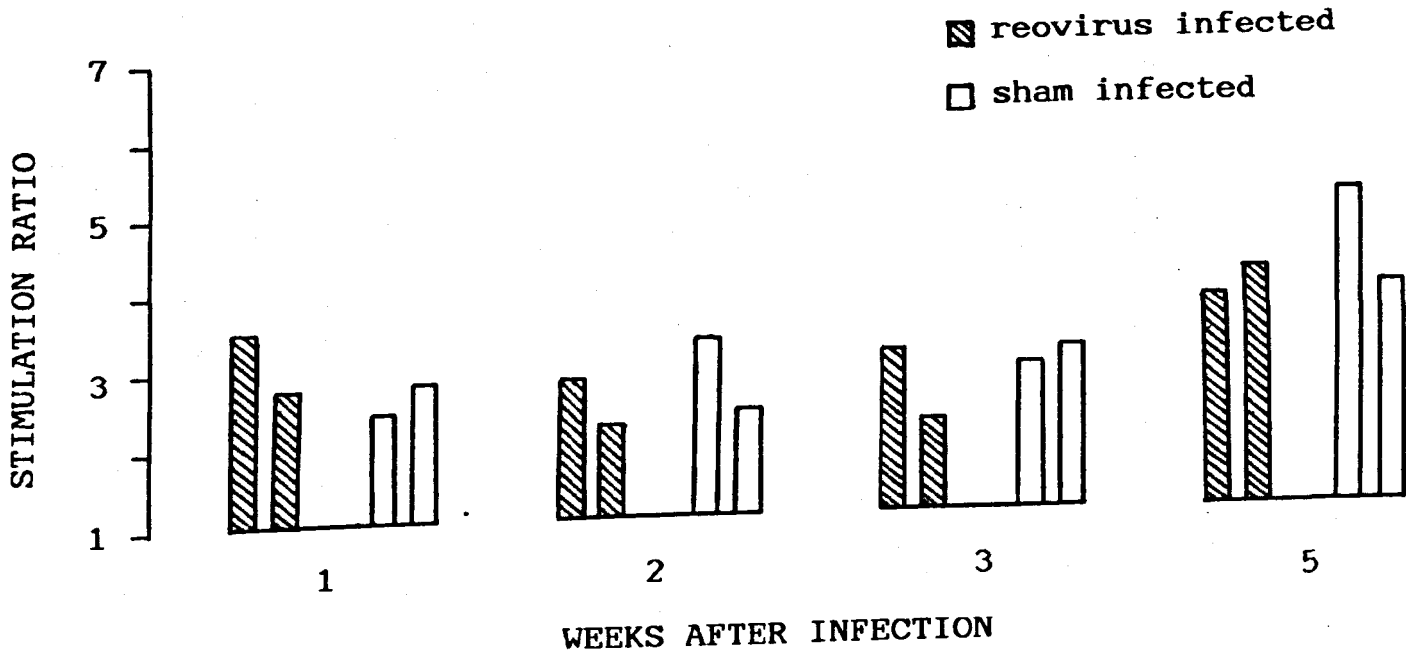

Fig. VI.5. Responses of peripheral blood lymphocytes of chickens to phyto-haemagglutinin following oral infection with reovirus or sham-infection at 1-day of age.

The peripheral blood lymphocytes of reovirus-infected and sham-infected chickens did not respond to the viral antigen in the present assay system.

4. DISCUSSION

The results of AGP tests obtained in the present study were similar to those observed by Jones and Kibenge (1984). Following infection with reovirus at 1-day of age, only one chick (infected by the foot-pad route) showed a positive AGP reaction at 2 weeks p.i., a large proportion became positive at 3 weeks p.i. but all were positive by the AGP test at 4 weeks p.i. However, Mukiibi-Muka et al (1984) found an earlier AGP reaction in chickens infected at 3 weeks of age: most of the chickens were positive for reovirus-antibody by the AGP test at 2 weeks p.i.

The neutralizing antibody responses observed in this study following reovirus infection were, in general, similar to those reported by Jones and Kibenge (1984) and Mukiibi-Muka et al, (1984). It would appear that although there was no striking difference in neutralizing antibody responses of chickens infected with reovirus by different routes (Fig. VI.1), the seroconversion was delayed in chicks

infected by oral or intra-articular route as compared with the foot-pad and subcutaneous routes. Jones and Kibenge (1984) also reported that, following oral infection, the SPF light breed chicks seroconverted predominantly at 3 weeks p.i. Mukiibi-Muka et al, (1984), using 3-weeks old chickens, observed a weak neutralizing antibody response to reovirus following subcutaneous inoculation as compared with the response after intramuscular, eye-drop and oral inoculation. However, in the present study the neutralizing antibody responses of younger chicks following subcutaneous inoculation were similar to those which occured after infection by the foot-pad route.

When the ELISA antibody titres of reovirus-infected chicks (Experiment 2) were compared with those of age-matched sham-infected chicks, the seroconversion was first noticed at 2 weeks p.i. (Fig. VI.2). In this experiment the PNT baseline titre was found to be 1:28 when determined as the mean plus twice the standard deviation of the ELISA titres of all sham-infected chickens. However, if the PNT baseline titre is determined from the ELISA titres of uninfected SPF mature chickens it would appear to be around 1:100 (Chapter III, Part 2). This may be due to the presence of some non-specific cross-reacting antibodies in the sera of mature chickens. If this titre (1:100) is selected as the baseline, the seroconversion in Experiment 2 would not be evident until 3 weeks p.i.

The DTH responses of reovirus-infected chickens to the viral antigen is very interesting. Although the test was done on a single occasion (at 6 weeks p.i.) using only one dilution of antigen, the positive results and the extent of the reactions observed would clearly indicate that chickens developed a cell mediated immune response following reovirus infection. DTH has also been reported to occur in mice following infection and subsequent challenge with mammalian reoviruses, and such a reaction was found to be serotype-specific (Weiner et al., 1980). The serotype specificity of DTH reactions in chickens infected with avian reovirus has not yet been recorded.

Since its original development (Mosmann, 1983), the colorimetric MTT assay has been used for evaluating lymphocyte transformation using sheep (Rai-el-Balhaa et al., 1985), monkey (Tatsumi and Yabe, 1986) and chicken (Konagaya and Oki, 1986) lymphocytes. In the present study, the results of the limited trials of the colorimetric LTA using PHA as mitogen would support the findings of other investigators, that this colorimetric assay could be useful as a simple and practical method for evaluating the activity of stimulated lymphocytes. The optical density of the blue formazan product is ideally measured at 570 nm. However, because of the unavailability of that particular wavelength filter in the author's laboratory, a lower wavelength was used during this

study, and this might have affected the results to some extent, by reducing the difference between the absorbances of blue formazan and the residual pale-yellow MTT. One advantage of this assay is that it avoids the need to use radioisotope. Although the results obtained by the MTT assay and the conventional radioisotope incorporation assay are not directly comparable, a very good correlation between these two tests was observed by Rai-el-Balhaa et al. (1985).

The assay system used in the present study was not fully standardized. The optimum concentration of PHA was determined by titration, but the cell concentration and incubation time were selected as those reported to be optimum for avian system by other investigators (Lee, 1974; Maheswaran, 1975).

In the initial trials, it was noticed that cells without mitogen also cleaved MTT to some extent and there was variation among lymphocytes from different chicks. However, the amount of formazan produced due to mitogen stimulation was proportional to that of unstimulated cells (Fig. VI.4). Therefore, the results of the subsequent tests are presented as the stimulation ratio.

In the present study the peripheral blood lymphocytes of reovirus-infected and uninfected control chickens did not show any significant difference in their ability to respond to PHA. However, Montgomery et al. (1986) observed a significant suppression

of the responses of lymphocytes of reovirus-infected chickens to PHA at 1 week p.i. but not at 3 weeks p.i. Further studies involving larger numbers of weekly samples are necessary to satisfactorily evaluate the effects of avian reovirus on the immunocompetence of chickens.

The failure to induce lymphocyte transformation with the viral antigen could have been due to the lack of optimum test conditions. Further standardization of the test should be attempted, using splenic lymphocytes, as well as, peripheral blood lymphocytes, longer incubation time (Scriba, 1974), a wider range of antigen dilution and various types of antigen preparation (Bloom and Rager-Zisman, 1975).

Part 2

EFFECTS OF PASSIVE IMMUNITY ON THE REPLICATION AND SPREAD OF REOVIRUS IN CHICKS

1. INTRODUCTION

Chicks hatched from eggs laid by hens immune to reovirus usually receive maternal antibodies from their yolk. This phenomenon has been used to protect young chicks by breeder vaccination (Cessi and Lombardini, 1975). Reovirus vaccines are now commercially available in many parts of the world. The efficacy of such vaccines is usually evaluated by their ability to protect the progeny of vaccinated flocks against the development of reovirus-induced tenosynovitis. However, it is not clear which stage of reovirus pathogenesis may be affected by the presence of maternal antibodies. The present part of this chapter describes the development of virus neutralizing and ELISA antibodies to reovirus in breeder chickens following vaccination, the correlation between VN and ELISA antibody titre and the effects of maternal antibodies on the replication and spread of avian reovirus in newly hatched chicks.

2. MATERIALS AND METHODS

2.1. Experimental design

This experiment was originally designed to investigate the role of maternal antibodies in protection against reovirus infection in newly hatched chicks, and in particular to establish a correlation between the levels of antibodies in hens and individual progeny chicks and the level of protection. The experimental design included, (i) vaccination of a group of breeder chickens with reovirus and monitoring their antibody responses individually, (ii) comparison of the extent of viral replication and spread in chicks obtained from vaccinated and unvaccinated hens. Unfortunately, the second part of the experiment had to be abandoned due to insufficient egg production by the vaccinated hens. Instead, an alternative, but less controlled experimental approach was followed as detailed below.

2.2. Vaccination of breeder chickens

Nine pairs of mature SPF hens and cockerels were held in isolation in 9 separate wire cages. All hens were inoculated subcutaneously in the cervical region with live reovirus R2 at a dose of 1 ml containing 10^6 $TCID_{50}$ per bird. Three weeks later, the same dose of virus, but inactivated with 0.1% formalin for 24 hours at 37°C and emulsified in equal

volume of Freund's complete adjuvant, was given to each hen subcutaneously. The hens were swabbed from the cloaca and bled from the wing vein weekly before and after vaccination until 5 weeks post vaccination. Virus isolation was attempted from the cloacal swabs, and the serum samples were tested by VN and ELISA as described in Chapter II (Section 4.2 and Section 5, respectively). A linear regression analysis was performed using log-transformed values of VN and ELISA titres to examine their correlation.

2.3. Viral replication and spread in passively immune and non-immune chicks

Thirty 1-day old SPF chicks were randomly divided into 10 groups, each consisting of 3 birds, and housed in 10 small cages. Five groups were infected orally and the rest were infected by the foot-pad route with 0.3 ml and 0.03 ml, respectively of various dilutions of a suspension of reovirus R2. The amount of virus per inoculum varied by $\log_{10}$ steps from 10^0 to 10^4 $TCID_{50}$ for both routes of infection. On day 4 p.i. the chicks were bled by cardiac puncture and killed by cervical dislocation. The carcases were opened aseptically and small pieces of tissues were collected from the hock joint, liver and middle jejunum, starting with the birds in the group infected with the lowest dose of virus. Special precautions were taken to avoid tissue-to-tissue contamination. Similar tissue

samples obtained from 3 birds of each group were pooled, weighed and stored at -70°C until used. Serum samples were also pooled from each group and stored at -70°C.

On a separate occasion, 30 one-day old chicks of a light egg laying strain were obtained from a commercial chicken flock which had been found to be seropositive to reovirus when serum samples from 10 randomly selected hens were tested for antibodies to reovirus by the ELISA method. The experimental protocol described above for the SPF chicks was repeated for these commercially obtained chicks.

The serum samples obtained from the chicks were tested by ELISA. The tissue samples were processed, and the amounts of virus were titrated as described in Chapter IV, Experiment 1 (Section 2.1.3).

3. RESULTS

3.1. Antibody responses of breeder chickens following vaccination

ELISA and VN antibody responses of 9 individual hens following vaccination are shown in Fig. VI.6. There was a sharp rise in antibody responses at 1 week after vaccination. In a few birds, the antibody level dropped slightly 3 weeks post vaccination,

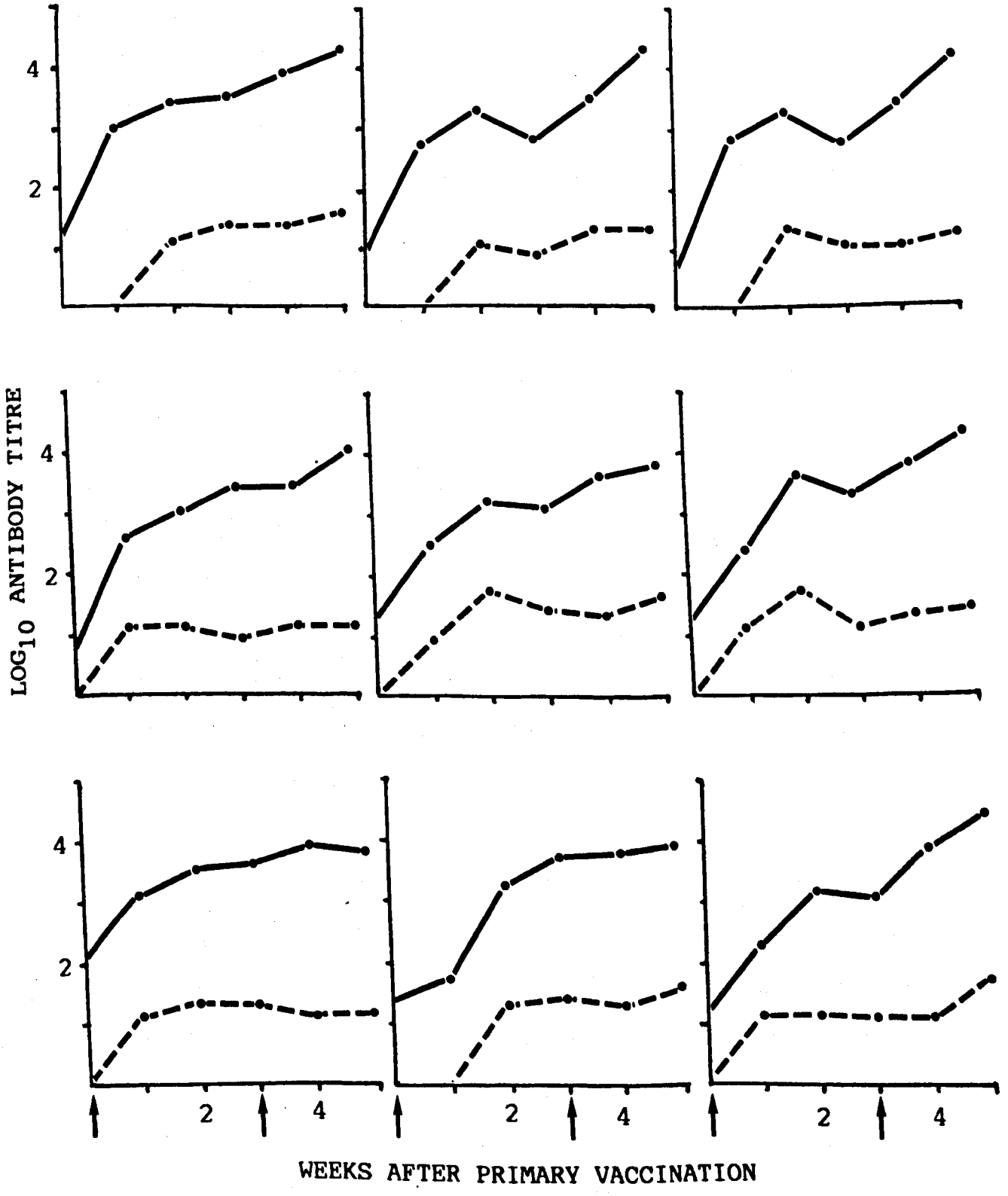

Fig. VI.6. ELISA (solid lines) and virus neutralizing (broken lines) antibody titres in the sera of 9 hens after vaccination with reovirus. Arrows indicate the time of primary and secondary vaccination.

but rose again in subsequent weeks following the boost given 3 weeks after primary vaccination. Although the overall neutralizing antibody titres were very low compared with the ELISA antibody, there was a significant positive correlation ($r=0.53$, $n=41$; $P<0.01$) between ELISA and neutralizing antibody titres as shown in Fig. VI.7.

3.2. Virus shedding following primary vaccination

The results of virus isolation from cloacal swabs are shown in Table VI.2. Of 9 hens, 7 shed virus at 1 week but only two birds did so at 2 weeks post vaccination. No virus was isolated from the cloacal swabs obtained at 3 weeks post vaccination or beyond. One hen (No.5) did not shed virus at any stage although she responded serologically in the same way as did the others.

3.3. Passive immunity and viral replication in chicks

The levels of maternal antibodies and the titres of virus in selected tissues of SPF and commercially obtained chicks after oral or foot-pad infection with various doses of the virus are shown in Tables VI.3 and VI.4. In the SPF chicks graded viral titres in tissues were obtained with various doses of viral inoculum. Although the virus was found in all tissues, the maximum amounts were in the jejunum following infection by either route. The ELISA titres of maternal

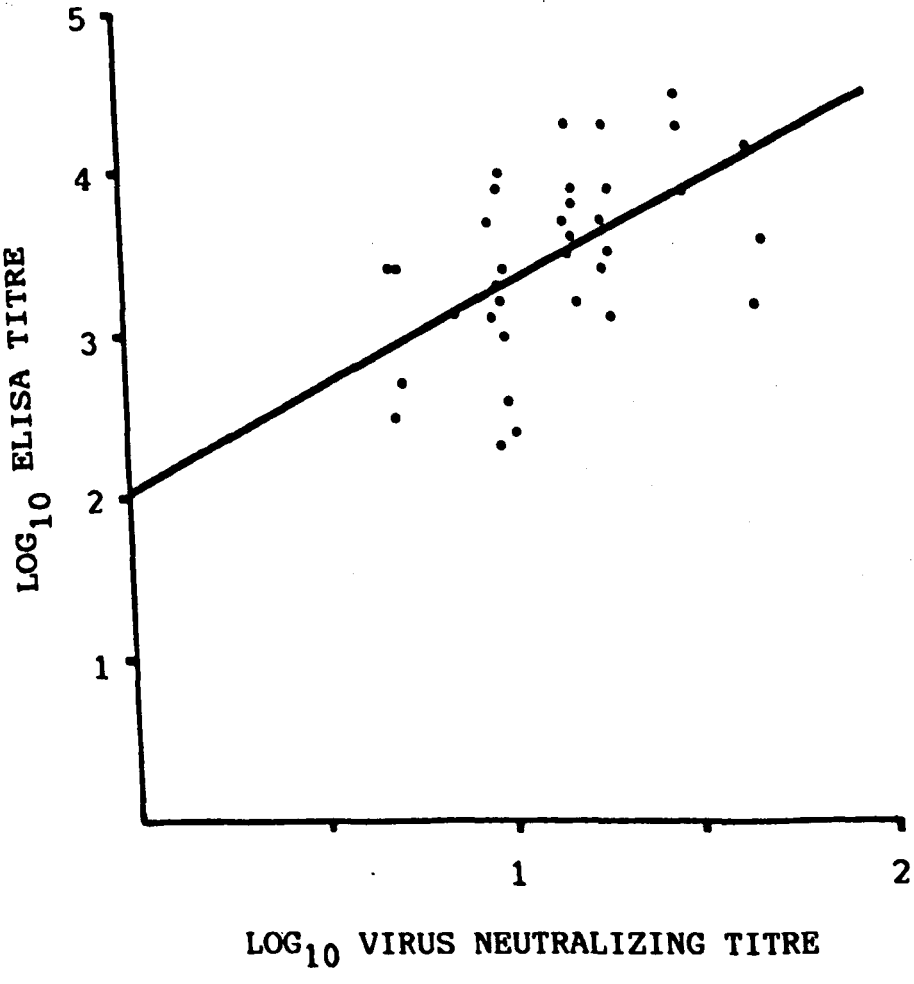

Fig. VI.7. Correlation between ELISA and virus neutralizing titres against reovirus (r=0.53, n=41; $P < 0.01$).

Table VI.2. Shedding of virus by mature hens following subcutaneous inoculation of live reovirus in emulsion

Bird identity	Weeks after primary vaccination				
	1	2	3	4	5
1	-	+	-	-	-
2	+	+	-	-	-
3	+	-	-	-	-
4	+	-	-	-	-
5	-	-	-	-	-
6	+	-	-	-	-
7	+	-	-	-	-
8	+	-	-	-	-
9	+	-	-	-	-

'+' positive for virus isolation

'-' negative for virus isolation in 3 subsequent blind passages.

Table VI.3. The levels of maternal antibodies and viral titres in tissues of SPF and commercial chicks 4 days after challenge with various doses of reovirus by the oral route at 1-day of age

Virus inoculum ($Log_{10}TCID_{50}$ per bird)	SPF chicks				Commercial chicks			
	Maternal antibody level (ELISA titre)*	Viral titre in tissue ($Log_{10}TCID_{50}$/g. tissue)			Maternal antibody level (ELISA titre)*	Viral titre in tissue ($Log_{10}TCID_{50}$/g. tissue)		
		Jejunum	Liver	Hock joint		Jejunum	Liver	Hock joint
4	1:20**	5.7**	3.8	3.2	1:184	5.8	-	-
3	1:26	5.6	3.1	2.9	1:219	5.3	-	-
2	1:27	5.1	2.3	+	1:128	6.1	-	-
1	1:18	4.7	+	+	1:250	4.3	-	-
0	1:13	4.8	2.3	+	1:169	4.1	-	-

* The geometric mean ELISA antibody titres in the parent flocks of the SPF and commercial chicks were 1:28 and 1:3,812, respectively.

** Antibody or virus titre of a pool of 3 samples.

'+' indicates the presence of virus at a titre (less than 2.3 $log_{10}TCID_{50}$/g. tissue) which could not be titrated.

'-' denotes negative for virus isolation in 3 subsequent passages.

Table VI.4. The levels of maternal antibodies and viral titres in tissues of SPF and commercial chicks 4 days after challenge with various doses of reovirus by the foot-pad route at 1-day of age

Virus inoculum ($Log_{10}TCID_{50}$ per bird)	SPF chicks				Commercial chicks			
	Maternal antibody level (ELISA titre)*	Viral titre in tissue ($Log_{10}TCID_{50}$/g. tissue)			Maternal antibody level (ELISA titre)*	Viral titre in tissue ($Log_{10}TCID_{50}$/g. tissue)		
		Jejunum	Liver	Hock joint		Jejunum	Liver	Hock joint
4	1:27**	5.7**	3.8	3.8	1:249	+	+	+
3	1:12	5.5	4.1	3.5	1:130	+	-	-
2	1:19	3.4	+	2.3	1:191	+	-	-
1	1:19	3.2	+	+	1:102	+	-	-
0	1:24	3.2	+	+	1:243	+	-	-

* The geometric mean ELISA antibody titres in the parent flocks of the SPF and commercial chicks were 1:28 and 1:3,812, respectively.

** Antibody or virus titre of a pool of 3 samples.

'+' indicates the presence of virus at a titre (less than 2.3 $log_{10}TCID_{50}$/g. tissue) which could not be titrated.

'-' denotes negative for virus isolation in 3 subsequent passages.

antibodies to reovirus in SPF chicks were between 1:12 and 1:27, which are well below the PNT base line ELISA titre for young chicks (Fig VI.2) and thus may be considered as non-specific. The ELISA antibody titres in commercial chicks were within a range of 1:102 to 1:250. The viral titres observed in the jejunum of commercial chicks following oral infection were similar to those found in the SPF chicks, however, no virus could be isolated from the liver or hock joint. Following infection by the foot-pad route, trace amounts of virus were found in the jejunum of commercial chicks, but no virus could be isolated from the liver and hock joint except in those which were infected with the largest dose of virus.

4. DISCUSSION

It was disappointing that following vaccination the hens did not lay sufficient eggs for the trial to be completed satisfactorily. Three hens stopped laying completely and others laid very infrequently. It was not known whether the drop in egg production was due to the social stress resulting from the isolation of hens in separate cages or to the effect of virus inoculation. Rau et al. (1980) observed

an unexplained transient drop in egg production in breeder chickens during the second week after vaccination. Dhillon et al. (1986) reported a drop in egg production and poor hatchability for a period of 7-8 weeks in a commercial poultry farm experiencing outbreaks of reovirus-tenosynovitis. However, further studies would be necessary to investigate the possible association between reovirus infection and the drop in egg production of laying chickens.

Serological responses to vaccination were evident in all hens as early as one week after vaccination. The ELISA antibody titres were very high in all birds. In contrast, the neutralizing antibody titres were very low, even lower than those observed in chicks following infection when 1-day old (Fig. VI.1). Using 3-weeks old chickens, Mukiibi-Muka et al. (1984) found a 2 to 4-fold lower neutralizing antibody titres after infection by the subcutaneous route than by other routes. It is not known, whether the lower neutralizing antibody responses as observed in the present study were due to the route of infection or the restricted replication of the virus in mature chickens, as indicated by the limited virus shedding.

Although there was a significant positive correlation between the titres obtained by ELISA and VN tests, the relative importance of these two tests in indicating the level of protection is yet to be determined.

The ELISA antibody titres of the random samples from the commercial parent flock and the progeny chicks indicate that the chicks received maternal antibodies from the egg yolk. The high viral titres found in the jejunum of SPF chicks following infection by both oral and foot-pad routes would support the findings of Chapter IV that for avian reovirus the intestine is a site of primary replication. It is of interest that following oral infection of commercial chicks the virus successfully replicated in the intestine but failed to reach target organs. This would indicate that the maternal antibodies neutralized circulating virus, and that the antibodies presumably were specific for the inoculated virus or at least partially so. This was further supported by the results of the foot-pad infection in commercial chicks, where only small amounts of virus were successful in reaching the intestine to commence their primary replication. A small amount of virus was able to localize in the hock joint and liver after infection with the highest dose only.

Although the relationship between the challenge virus and the virus to which the commercial chickens had their antibodies has not been established, the maternal antibodies were apparently effective in preventing the challenge virus from localising in the target tissues.

The ability to complete productive replication in the intestine of even maternally immune subjects and to be shed into the gut lumen probably allows reoviruses to survive in nature and to enjoy a ubiquitous distribution.

Part 3

EXPERIMENTAL REOVIRUS INFECTION AND AUTOIMMUNITY IN CHICKENS: A PRELIMINARY STUDY

1. INTRODUCTION

The lesions of reovirus-induced tenosynovitis or viral arthritis of chickens have some striking similarities to those of immune-based arthritis in other species including human rheumatoid disease. Several types of autoantibodies have been found to be associated with human rheumatoid arthritis including rheumatoid factor (RF), an autoantibody directed against IgG (Franklin et al., 1957), antinuclear antibodies (ANA) (Ward et al., 1964) and anti-collagen antibodies (ACA) (Steffen and Timpl, 1963). The auto-antibodies can form immune complexes which may circulate in blood and become deposited in the synovium, which in turn may lead to the development of arthritis.

Attempts to demonstrate RF in the sera of chickens infected with reovirus have been unsuccessful (Taylor, 1965; Walker, 1975) but the presense of ANA has been reported by Pradhan et al. (1987).

The present part of this chapter describes the results of some preliminary studies on the possible association of autoantibodies with experimental reovirus infection in chickens.

2. MATERIALS AND METHODS

2.1. Experimental design

Two batches of sera collected from chickens after experimental infection with reovirus (see below) were examined in the following investigations:

a) Search for rheumatoid factor,

b) Examination for anti-collagen antibodies and comparison with experimentally induced anti-collagen antibodies,

c) Examination for antinuclear antibodies, and

d) Precipitation and analysis of immune complexes; comparison of these complexes with virus-antibody complexes prepared *in vitro*.

2.2. Serum samples

The serum samples used in this part of the study were obtained from two sources:

i) The first batch was obtained from a previous experiment detailed in Part 1 (Experiment 2) of this chapter. They were collected from chicks 1-6 weeks after oral infection with reovirus or oral sham-inoculation with uninfected CELi cell culture fluid at 1-day of age. No clinical disease or gross lesions developed in these chicks but microscopic lesions of tenosynovitis were found at the end of the experiment (8 weeks p.i.).

ii) The second batch comprised eight serum samples which were obtained from Mr. A. Afaleq. These sera

were collected 3 weeks after infection of day-old chicks by the foot-pad route with each of 3 strains (R2, R11 and R19) of chicken reovirus. Gross and microscopic lesions of tenosynovitis and cartilage lesions as described in Chapter V were observed in these chicks at 3 weeks p.i.

2.3. Test for IgM rheumatoid factor

A multiple layer indirect ELISA was used in an attempt to detect IgM-antiIgG rheumatoid factor in the sera of reovirus-infected chickens. The wells of PVC microtitre plates were coated with chicken IgG* (10 µg/ml) by overnight incubation at 4°C. Then a 1:25 dilution of each serum sample was added to triplicate wells. This was followed by the addition of a 1:50 dilution of monoclonal mouse-antichicken IgM (kindly supplied by Dr. A.P.A. Mockett), a 1:2000 dilution of peroxidase-conjugated sheep-antimouse IgG(H+L)** and then OPD substrate solution. All reagents were added in 50 ul volume per well, and between each step the excess reagents were removed by washing. All reagents, except the antigen (IgG), were incubated for 1 hour at 37°C. The diluents, washing buffer and substrate solution were the same as those used in the ELISA for detecting reovirus-antibody (Chapter II, Section 5.3). The plates were read at a wavelength of 450 nm.

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* Nordic Immunological Lab. Ltd., Tilburg,The Netherlands.

** Sera-Lab Ltd., Sussex, England.

2.4. Experimental induction of anti-collagen antibodies in chickens

2.4.1. Collagen

A 99% pure lyophilized preparation of chick type II collagen* was dissolved in 0.1N acetic acid at a concentration of 1 mg per ml by overnight stirring at 4°C. The collagen solution was used either immediately or stored frozen in aliquots at -70°C.

2.4.2. Inoculation of chickens with collagen

Ten 5-weeks old SPF cockerels were used. The collagen inoculum was prepared by emulsifying freshly dissolved collagen with an equal volume of Freund's complete adjuvant. The sham inoculum was prepared in the same manner by emulsifying 0.1N acetic acid with the adjuvant. Three ml of collagen emulsion (1.5 mg collagen) was inoculated subcutaneously at 3 to 4 sites around the cervical and thoracic regions of each of 6 birds. The remaining 4 birds were sham-inoculated with acetic acid emulsion in the same manner. The birds were kept under close observation. All birds were bled from the wing vein at 5 weeks after inoculation and killed by barbiturate euthanasia two weeks later. Post-mortem examination was conducted on them and tissues were collected from the hock joint for histological examination.

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* Koch-Light Ltd., Harverhill, Suffolk, England.

2.5. Test for anti-collagen antibody

An indirect ELISA was performed to detect the presence of ACA in the serum of collagen-inoculated, reovirus-infected and control chickens. The test protocol was essentially the same as that used for detecting reovirus antibody (Chapter II, Section 5.3) except that the plates were coated with collagen (10 µg/ml) instead of viral antigen by incubating overnight either at 4°C or at 37°C to obtain native or heat denatured collagen-coated plates, respectively. The serum samples were used at a 1:25 dilution.

2.6. Immunofluorescent staining for antinuclear antibody

Indirect immunofluorescence was used to detect antinuclear antibodies in the sera of chickens infected with reovirus. Preformed CELi or VERO cell cultures were used as the substrate. A few selected sera were also tested on cryostat sections of snap-frozen chicken liver. For the cell cultures, a drop (50 µl) of CELi or VERO cell suspension was placed in each well of a teflon-coated multiwell slide and incubated at 37°C in a humidified CO_2 incubator, until the cells became nearly confluent (usually 18 hours). Cell monolayers were rinsed briefly in PBS-A to remove all dead and unattached cells and then fixed in acetone for 5 minutes at room temperature. Cryostat sections were cut at 5 microns thickness from a snap-frozen tissue block of liver from a 5-week old chicken.

The sections were mounted on glass slides, air dried for 30 minutes and fixed in acetone for 5 minutes at room temperature. Following fixation the cell monolayers or sections were washed for 15 minutes in 3 changes of 0.01M PBS, pH 7.1. A 1:5 dilution of each serum sample was applied on cell monolayers or sections in duplicate and incubated for 1 hour at 37°C in a humidified box. After washing as above the cell monolayers or sections were covered with a 1:50 dilution of FITC-conjugated rabbit-antichicken IgG* and incubated for a further 30 minutes at 37°C. The specimens were finally washed as above and covered with non-fade mountant under coverslips. They were examined with a Leitz Ortholux fluorescence microscope equipped with UV/blue incident light.

Any sample found positive at a 1:5 dilution was titrated on CELi cell culture monolayers using serial two-fold dilutions.

2.7. Precipitation of immune complexes from the sera of reovirus-infected chicks

A 2.5% solution of polyethylene glycol (PEG) was used to precipitate immune complexes from serum. The protocol described by Hudson and Hay (1980) was used with some modification.

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* Nordic Immunological Lab. Ltd.,Tilburg,The Netherlands.

A working solution of PEG was prepared by mixing 6 ml of 25% PEG stock solution with 3 ml of 0.2M EDTA, pH 7.6 and 1 ml of veronal buffered saline (VBS, Appendix-A). Twenty microlitres of working PEG solution were added to 100 µl of each serum sample in microcentrifuge tubes to obtain a final 2.5% PEG concentration. They were mixed thoroughly, left overnight at 4°C and then centrifuged at 2000g for 20 minutes at 4°C in a bench centrifuge. The tubes were placed in ice, the supernatants were removed and the precipitates resuspended in 1.5 ml of 2.5% PEG solution in VBS containing 0.01M EDTA. They were centrifuged again as above, the supernatants were removed and the precipitates were finally redissolved in 100 µl of VBS containing 0.015M sodium azide and stored at 4°C until used for further analysis. They were incubated for 1 hour at 37°C immediately before use to ensure that the precipitates had redissolved.

2.8. Determination of IgG activity in immune complexes

The IgG activity in PEG precipitates of the serum samples was determined by a captured ELISA method. The wells of PVC microtitre plates were coated with Fc-specific goat-antichicken IgG* (10 µg/ml) by overnight incubation at 4°C. Triplicate 1:100 dilutions of each sample (PEG precipitate) were added to coated wells. This was followed by the addition

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* Nordic Immunological Lab. Ltd.,Tilburg,The Netherlands.

of a 1:2000 dilution of peroxidase-conjugated rabbit-antichicken IgG and then OPD substrate solution. All reagents were added in 50 µl volumes per well and between each step the unbound reagents were removed by washing. All reagents, except coating antibody were incubated for 1 hour at 37°C. The diluents, washing buffer and substrate solution were the same as those used in the ELISA for detecting reovirus-antibodies (Chapter II, Section 5.3). The plates were read at a wavelength of 450 nm.

2.9. In vitro preparation and precipitation of reovirus-antibody immune complex

Fifty microlitres of three dilutions of reovirus suspension containing 10^1, 10^3 and 10^5 $TCID_{50}$ were added in duplicate to microcentrifuge tubes, each containing 50 µl of either an anti-reovirus hyperimmune chicken serum or an SPF reovirus-negative chicken serum. In two sets of controls, either serum or virus was substituted by medium 199. After thorough mixing the tubes were incubated at 37°C for 2 hours to facilitate the formation of immune complexes. Then the complexes were precipitated from the virus-serum mixtures with 2.5% PEG and the IgG activity of the precipitates was measured as described above (Sections 2.7 and 2.8, respectively).

3. RESULTS

3.1. IgM rheumatoid factor

No IgM-antiIgG rheumatoid factor was detected in any of the sera of reovirus-infected chickens. Variations in the dilution and incubation time of different reagents did not reveal any RF undetected by the standard protocol.

3.2. The effects of collagen inoculation of chickens

One of the 6 collagen-inoculated chickens developed lameness with unilateral swelling of the hock joint at 4 weeks post inoculation. As the bird was unable to reach food and water, it was humanely killed at 5½ weeks after inoculation. All other collagen-inoculated and sham-inoculated birds remained normal until the end of the experiment (7 weeks post inoculation). On post mortem examination the clinically affected bird had a swollen hock joint containing gelatinous exudate between tendons in the hypotarsal sesamoid. All other birds appeared to be normal. Microscopic examination revealed lesions in the hock joint of another collagen-inoculated bird in addition to the clinically affected one. Microscopic changes were characterized by the presence of diffuse heterophilic and lymphocytic infiltration in the tendons and tendon sheaths of the hypotarsal sesamoid. In both cases colonies of gram-positive bacteria,

resembling staphylococci, were seen in some areas of the affected tendon sheaths, and this was confirmed by Gram-Humberstone stain.

3.3. Anti-collagen antibodies in chicken sera

The results of ELISA for detecting collagen autoantibodies in the sera of collagen-inoculated and reovirus-infected chickens are shown in Fig. VI.8. Five of 6 sera obtained from collagen-inoculated chickens gave positive reaction with both native and denatured collagen as compared with the sera collected from the sham-inoculated chickens. The remaining serum reacted with denatured collagen but not with native collagen. The absorbance values of both collagen-inoculated and sham-inoculated chicken sera were higher in the denatured collagen-coated plates.

The serum samples obtained from reovirus-infected chickens did not show strong positive reactions. However, if a PNT baseline is drawn as the mean plus twice the standard deviation of the absorbance values of all age-matched control chicken sera, it would appear that a small number of the sera obtained 6 weeks after oral infection and most of those obtained 3 weeks after foot-pad infection could be considered as positive for ACA.

However, the examination of a small number of sera from an SPF laying flock showed that these 'controls'

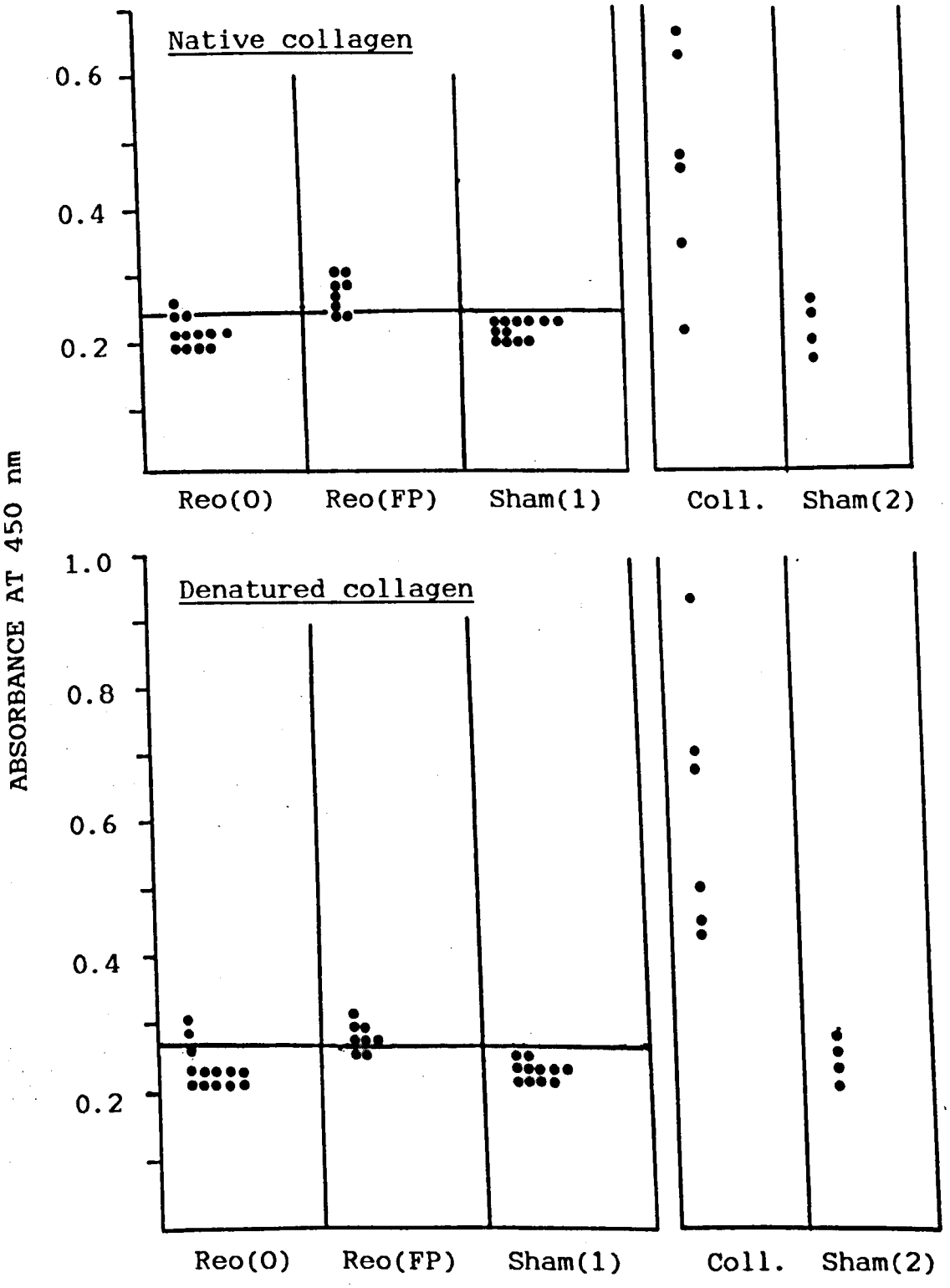

Fig. VI.8. Autoantibodies to native and denatured collagen, as measured by ELISA, in the sera of chickens obtained 1-6 weeks after oral [Reo(O)] or 3 weeks after foot-pad [Reo(FP)] infection with reovirus or 1-6 weeks after oral sham-infection [Sham(1)] given at 1-day of age; and 5 weeks after collagen inoculation [Coll.] or sham-inoculation [Sham(2)] given at 5 weeks of age. Horizontal PNT baselines were drawn as the mean plus twice the standard deviation of all samples in Sham(1).

had levels of ACA which were higher than those for the young control birds tested here. In that case the raised levels of ACA in the chicks experimentally infected with reovirus would not be evident.

3.4. Antinuclear antibodies in reovirus-infected chicken sera

The results of the indirect immunofluorescence tests for ANA are shown in Table VI.5. All 4 sera obtained from orally infected chickens on the last sampling occasion (6 weeks p.i.) were positive for ANA when tested on CELi cell monolayers. All other serum samples including those obtained from chickens 3 weeks after infection with reovirus by the foot-pad route were negative for ANA. Two of the 4 samples found positive for ANA on CELi cell monolayers were also positive on VERO cells and cryostat liver sections. When titrated on CELi cell monolayers, one serum had a titre of 1:64 and the titre of each of the remaining 3 sera was 1:16. The nuclear staining pattern in the CELi cell monolayers was mainly homogeneous (Fig. VI.9) but that in VERO cell monolayers had a mainly speckled appearance (Fig. VI.10). In cryostat liver sections the staining was mainly homogeneous but was less definite (Fig. VI.11).

3.5. IgG activity in immune complexes

The results of ELISA for IgG activity in PEG precipitates of the sera obtained from reovirus-infected

Table VI.5. Results of indirect immunofluorescence test using different substrates for antinuclear antibodies in the sera of chickens infected with reovirus at 1-day of age by the foot-pad or oral route

Substrate	Serum samples*	
	3 weeks p.i. (Foot-pad)	6 weeks p.i. (Oral)
Chicken embryo liver cell culture	0/8**	4/4 (1:64,1:16,1:16,1:16)
VERO cell culture	0/8	2/4 (NP)
Cryostat liver section	NP	2/4 (NP)

* Serum samples obtained 1-5 weeks after oral infection with reovirus and 1-6 weeks after sham-infection were negative for ANA when tested on CELi cell culture (not shown in table).

** No. of samples positive for ANA / No. examined. Figure in parenthesis indicate the titres of the positive samples.

NP. denotes not performed.

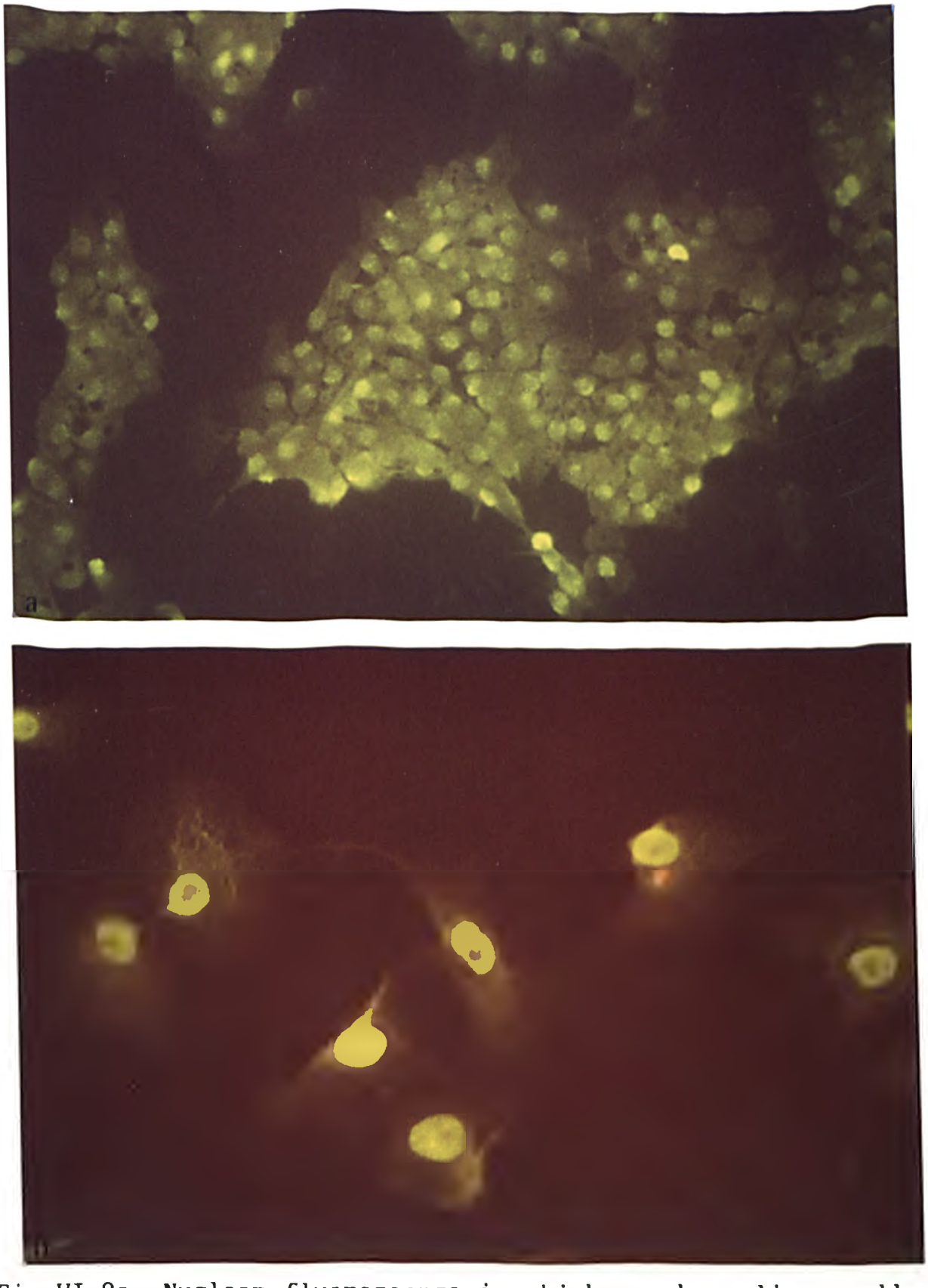

Fig.VI.9a. Nuclear fluorescence in chicken embryo liver cell culture indicating the presence of antinuclear antibody in the test serum. Indirect immunofluorescence using an FITC-conjugated antichicken IgG (X560).

b. Same as above showing few isolated cells at higher magnification. Staining of the nuclei is mainly homogeneous (X900).

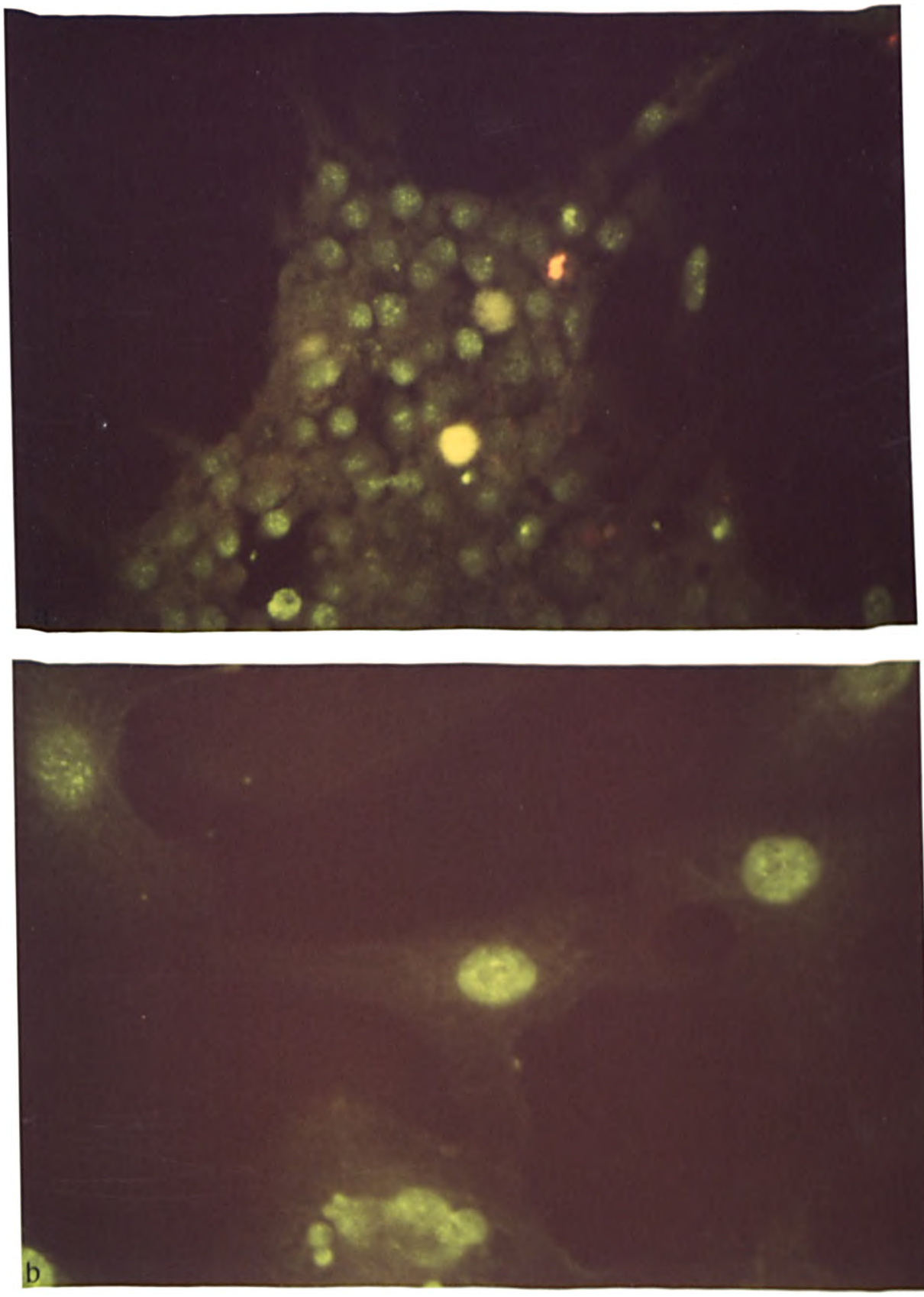

Fig.VI.10a. Nuclear staining by indirect immunofluorescence in VERO cell culture indicating the presence of antinuclear antibody in the test serum (X560).

b. Same as above showing a few isolated cells at higher magnification. Staining of the nuclei is mainly of the granular (speckled) type (X900).

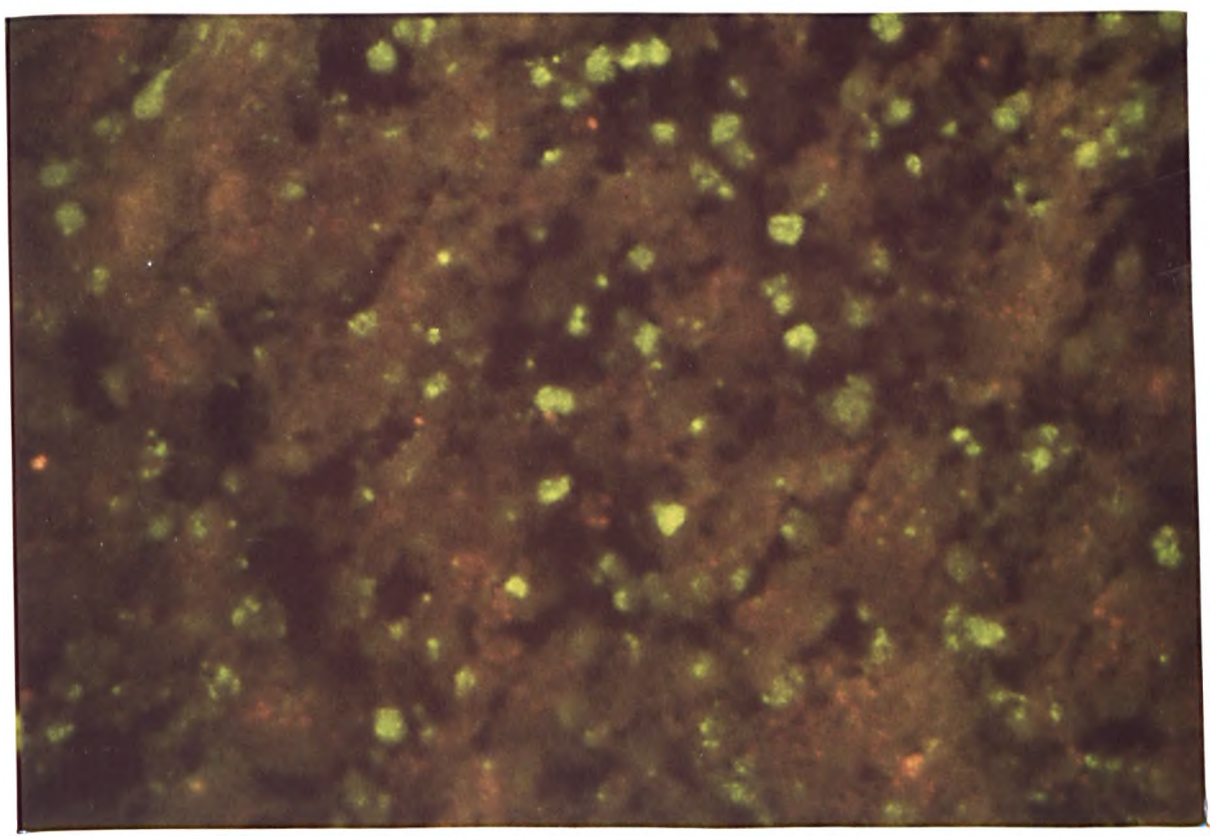

Fig.VI.11. Nuclear staining by indirect immunofluorescence in cryostat section of chicken liver indicating the presence of antinuclear antibody in the test serum. Staining of the nuclei is mainly of the homogeneous type (X560).

and sham- infected control chickens are shown in Fig. VI.12. The amounts of IgG precipitated by PEG from the serum samples of reovirus-infected chickens were consistently higher than those from uninfected control chickens during the period of 2 to 6 weeks p.i. There was little difference in the IgG concentration of immune complexes precipitated from the sera obtained from chickens 3 weeks after oral or foot-pad infection with reovirus.

The IgG activity in the PEG precipitates derived from <u>in vitro</u> mixtures of reovirus plus antiserum or normal serum is shown in Fig. VI.13. The amount of IgG precipitated by PEG from the anti-reovirus serum in the absence of virus was comparatively higher than that obtained from the normal serum. However, the IgG activity in the precipitates of normal serum remained consistent irrespective of the presence or absence of virus, while the precipitation of IgG from the anti-reovirus serum was higher and increased in the presence of increased concentration of virus upto 10^3 $TCID_{50}$.

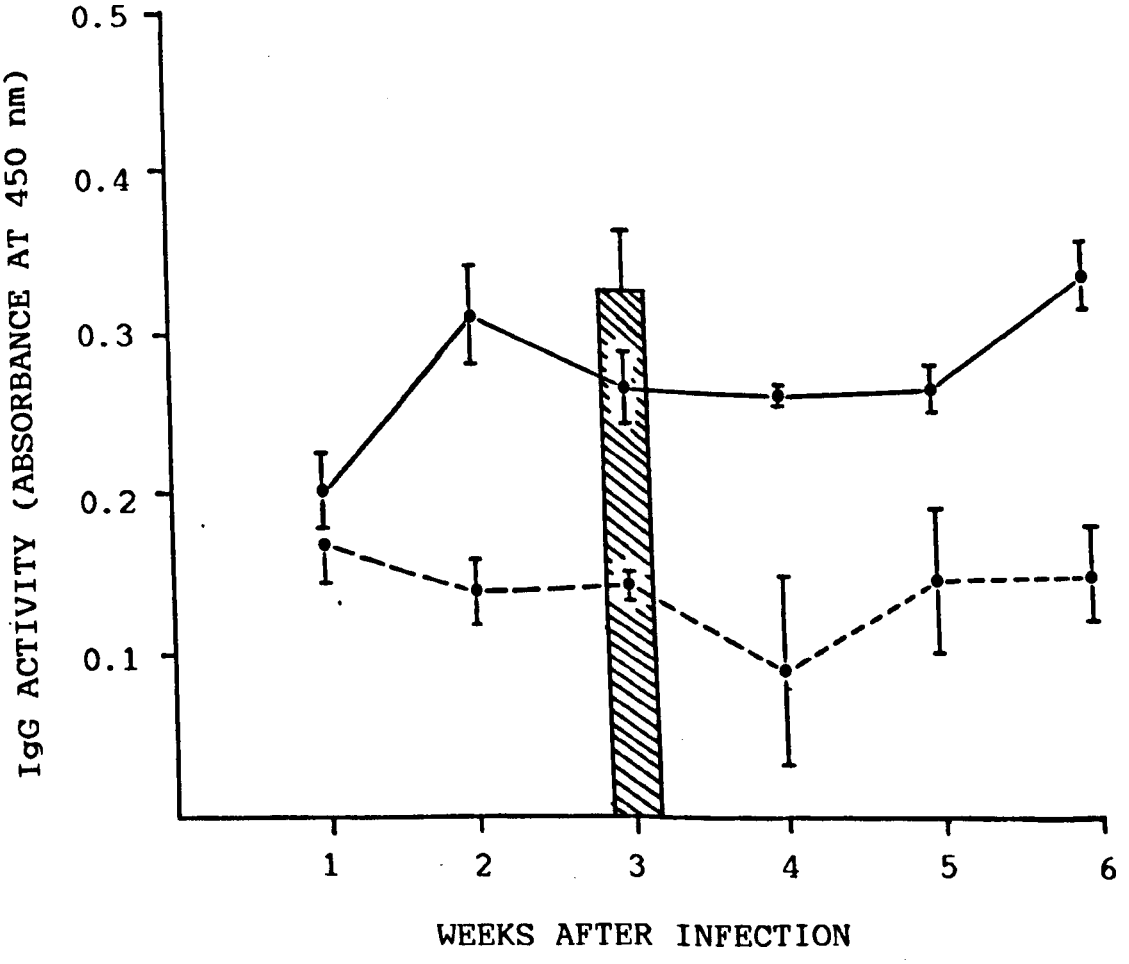

Fig. VI.12. Precipitation of immune complexes by PEG from sera of chickens infected orally (solid line) or by the foot-pad route (bar) with reovirus or sham-infected (broken line) when 1-day old. The figure shows IgG activity in the precipitates as measured by ELISA. Vertical line at each point represents standard deviation of replicate samples.

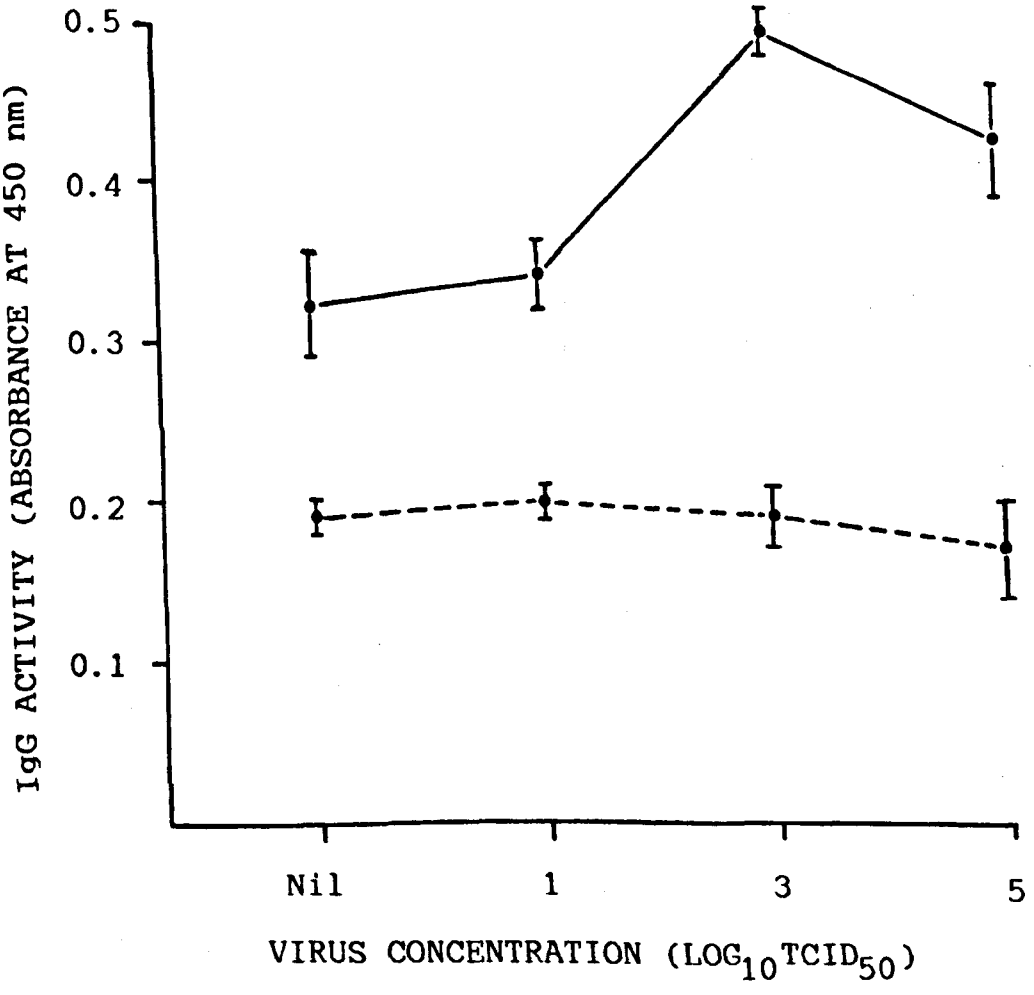

Fig. VI.13. Precipitation of immune complexes by PEG from the mixtures of different concentrations of reovirus plus homologous hyperimmune chicken serum (solid line) or normal chicken serum (broken line). The figure shows the IgG activity in PEG-precipitates as measured by ELISA. Vertical line at each point represents standard deviation of replicate samples.

4. DISCUSSION

The tissue damage in human rheumatoid arthritis is believed to be due to a type III hypersensitivity reaction mediated by an IgG-RF immune complex, while cell mediated (type IV) hypersensitivity may also be involved. It is also believed that RF is formed when an antigenic determinant is revealed on the Fc portion of the IgG molecule as it combines with an antigen which has not yet been identified (Taussig, 1979). Involvement of a virus or viruses in initiating human rheumatoid arthritis has been suggested (Marmion, 1976).

Reovirus-induced arthritis of chickens is a disease with a known aetiological agent and it shares some pathological features of human rheumatoid arthritis. The present study was undertaken to examine the hypothesis that avian viral arthritis might serve as a model for human rheumatoid arthritis.

The ELISA system used in this study to detect IgM RF was basically the same as used by Gripenberg et al. (1979) for demonstrating RF in human sera, although in the present system an unconjugated anti-IgM serum and then an appropriate conjugate were used instead of conjugated anti-IgM antibodies. However, no IgM RF could be detected in the sera of reovirus-infected chickens. This finding is in agreement with observations of Walker (1975) who was also unable

to detect any RF in chicken sera after reovirus infection using tanned cell haemagglutination and latex agglutination tests.

Although there is strong evidence that the RF is associated with rheumatoid arthritis, many rheumatoid patients do not have RF in their sera. Moreover, in addition to RF, some other autoantibodies, such as ANA, ACA are sometimes found in the sera of individuals with rheumatoid arthritis, and it is suggested that these autoantibodies could play an important role in the pathogenesis of the disease (Holborow and Swannell, 1977).

The presence of high levels of ACA in the sera of collagen-inoculated chickens would indicate that type II collagen can act as a potential autoimmunogen in chickens. The relationship between the collagen-inoculation and development of joint lesions, as observed in two chickens, could not be fully interpreted due to the presence of bacteria in the lesions. Several types of bacteria including and most commonly Staphylococcus aureus have been isolated from natural outbreaks of synovitis in chickens, but their role as a primary aetiological agent of synovitis has not been established (Kibenge and Wilcox, 1983). In the present study it remained unclear whether the chickens became infected with the bacteria spontaneously, or received them as a contaminant of the inoculum. A third possibility which may be the most likely is that the staphylococci

were endogenous and became involved in the joint as a result of the development of collagen-induced synovitis.

It would appear from the results of ELISA testing for ACA that most of the sera of the chicks infected with reovirus by the foot-pad route and a few of those infected orally had slightly higher levels of ACA as compared with the age-matched controls. However, the raised level of ACA in reovirus-infected chickens would not be apparent if compared with sera of much older control chickens. It is known that autoantibodies occur in all individuals to a greater or lesser extent, and the levels increase with age (Taussig, 1979). Thus in the interpretation of these findings for ACA following reovirus infection, it is important that age-matched controls be used. The immunopathological significance of ACA in reovirus-infected chickens will remain unclear until further studies are carried out.

The presence of ANA in reovirus-infected chickens is interesting. In mammalian species, ANA is the characteristic of systemic lupus erythematosus, but it is also found less frequently in rheumatoid arthritis and at a low titre in a proportion of normal individuals (Holborow and Swannell, 1977). Adair and Calvert (1980) demonstrated the presence of ANA in a small percentage of normal chicken sera. In the present study, ANA was detected in chickens 6 weeks after

oral infection with reovirus. Despite the presence of more severe lesions, ANA could not be detected in the sera obtained from chickens 3 weeks after foot-pad infection. However, Pradhan et al. (1987) after infection by the foot-pad route were able to detect ANA in a proportion of chickens at 3 and 4 weeks p.i. and in all chickens from 5 to 18 weeks p.i. They used cryostat sections of mouse liver as the substrate. In the present study CELi and VERO cell monolayers and cryostat sections of chicken liver were used. Both cell culture systems appeared to give better definition of positive staining than the cryostat liver sections. Further studies will be needed to determine their relative sensitivity. Homogeneous or speckled type nuclear staining pattern noticed in homologous CELi cells and mammalian VERO cells, respectively merits further study as the different types of nuclear staining are believed to represent reactions with different nuclear antigens.

The reason(s) for the appearance of various autoantibodies in individuals is not clear. Several mechanisms have been suggested for the development of virus-induced autoimmunity (Hirsch and Proffitt, 1975). These include: (a) release of 'sequestered' antigens from virus-infected cells or tissues, (b) virus-induced alteration of host cell membrane antigen, (c) immunologic crossreactivity between viral and host antigenic determinants, (d) direct or indirect

alteration of immunocytes and/or immune regulator cells by virus infection of those cells.

The presence of autoantibody in a disease condition does not necessarily establish the involvement of the autoantibody in the pathogenesis of the disease. If an autoantibody plays any role in the pathogenesis of the disease concerned, it may be mediated by immune complex formation with the autoantigen and a subsequent type III hypersensitivity reaction.

The PEG precipitation technique, used for the detection of circulating immune complexes, was based on the observation that PEG at lower concentration precipitates IgG mainly as a part of immune complexes, while minimal free IgG is precipitated (Creighton et al.,1973). Comparatively higher amounts of IgG were precipitated from the sera of chickens infected with reovirus than from normal chicken sera. This would suggest that immune complexes may occur in reovirus-infected chickens, although the antigenic component of these complexes is not known. The in vitro study using a mixture of reovirus and antiserum indicated that reovirus-antibody complexes can be precipitated by 2.5% PEG. Further studies are necessary to examine the composition of circulating immune complexes of reovirus-infected chickens and the possibility of these complexes being deposited in the synovium and fixing complement to induce type III hypersensitivity-mediated tissue damage.

Experiments described in this part of the thesis must be considered as preliminary ones since they were conducted on a relatively small number of sera from young birds which had not been allowed to develop severe lesions of tenosynovitis or arthritis. However, the results provide some evidence for the involvement of autoimmunity in reovirus infection.

Cell-mediated autoimmune reactions might also be important in the pathogenesis of reovirus-induced arthritis. Rheumatoid arthritis can occur in congenitally agammaglobulinaemic boys, where autoantibodies presumably play no role at all (Taussig, 1979). Further detailed investigations on the involvement of humoral and cellular autoimmune reactions in reovirus infection would be necessary to implicate autoimmune mechanisms in reovirus-induced arthritis in chickens.

Chapter VII

GENERAL DISCUSSION AND CONCLUSION

A general scheme for studying the pathogenesis of a microbial infection was originally proposed by Fenner (1948) and Mims (1964), and has been followed by Fields and his co-workers for investigating the pathogenesis of mammalian reovirus infection (Sharpe and Fields, 1985; Tyler and Fields, 1986b). This approach is based on the attempts to explore the following events of viral pathogenesis (Kauffman and Fields, 1986):

a) viral entry into host,
b) primary replication,
c) viral spread within host,
d) cell and tissue tropism,
e) host immune response and other host defence factors,
f) tissue injury, and
g) viral persistence.

In the investigations described in this thesis a similar approach was followed to improve our understanding of the pathogenesis of avian reovirus infection.

From what is already known, augmented by findings of this thesis the major events in the pathogenesis

of reovirus-induced tenosynovitis may be depicted diagrammatically (Fig. VII.1).

It would appear from the results of the early pathogenesis studies (Chapter IV) that intestinal and bursal epithelial cells of chickens serve as the portal of entry for avian reovirus. The results of the electron microscopic study (Chapter IV) would indicate that the virus probably enters the cell through a process of receptor-mediated endocytosis. The entry of virus is quickly followed by primary replication in the intestine and in the bursa of Fabricius as indicated by a rise in viral titre and positive FA and IP staining of viral antigen within 12 to 24 hours of infection.

The result of this study also suggested that, in addition to the active penetration of epithelial cells, which is naturally followed by viral uncoating and replication, the virus may also be transported passively across the epithelial barrier, as indicated by the presence of virus in the liver and kidney as early as 6 hours after infection. The passive transportation of avian reovirus could take place in the bursa of Fabricius where follicle-associated epithelial cells or M cells are actively involved in macromolecular pinocytosis and transport. If this is so, then it resembles, in part, mammalian reovirus infection in mice where the virus enters the intestine through the M cells located over the ileal Peyer's patches (Wolf et al., 1983).

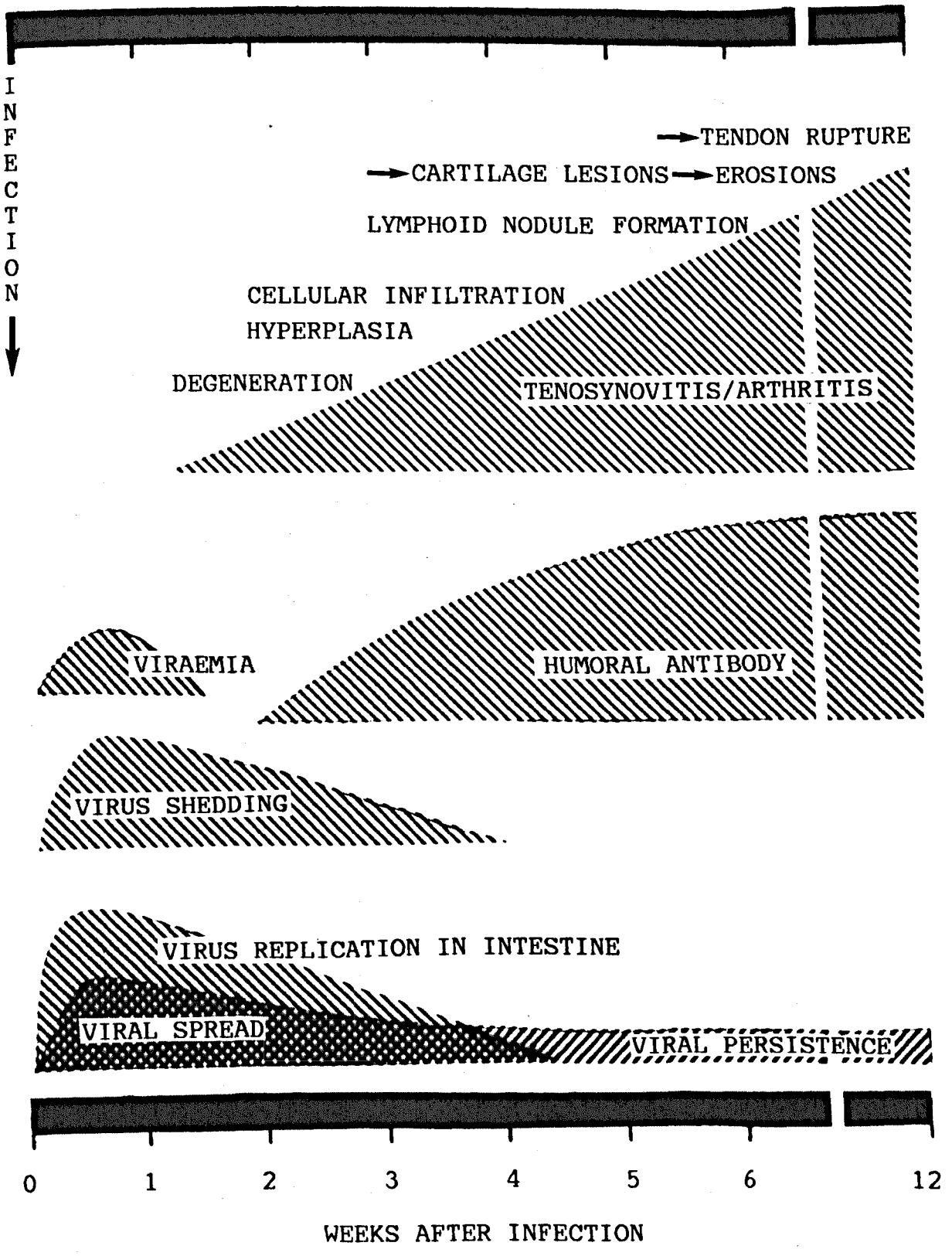

Fig. VII.1. Schematic representation of the major events in the pathogenesis of reovirus infection in chickens.

The systemic spread of reovirus in chicks following oral infection appears to be very rapid. The virus reaches most organs within 12 to 24 hours. Although major part of viraemia is associated with plasma (Kibenge et al., 1985), the possibility of lymphoid cell-associated transport cannot be dismissed without further study.

Following viraemia, avian reovirus, like its mammalian counterpart, appears to be pantropic in distribution during the early stage of infection. High viral titres are maintained in many tissues for several days, following which there is a gradual decline, probably due to the operation of host defence mechanisms.

Following infection at day-old, the cloacal virus shedding in chicks continues for 3 to 4 weeks (Chapter V), but the virus persists, at low levels in the hock joint for a long period of time as reported by other investigators (Marquardt et al., 1983; Jones and Kibenge, 1984).

Information on the immune response of chickens to reovirus is still very limited. Humoral antibodies can be detected 2 to 3 weeks after infection of day-old chicks. The antibody titres continue to rise for at least 5 to 6 weeks after infection (Chapter VI). There is no doubt that humoral antibodies can neutralize virus that is circulating in plasma and can protect chickens from reinfection. Maternal antibodies can

protect newly hatched chicks by preventing viral spread and seeding in target tissues (Chapter VI). However, it is unlikely that antibody itself can destroy intra-cellular persistent virus. Antibody-dependent cellular cytotoxity or complement-mediated lysis has not yet been reported in avian reovirus infection.

Almost nothing is known about cell-mediated immunity in chickens following reovirus infection. The delayed hypersensitivity reaction, as observed in the present study (Chapter VI) would indicate the involvement of a cell-mediated immune response. However, detailed studies would be necessary to investigate whether the cell-mediated immunity is effective enough to destroy persistent intra-cellular virus. Recently it has been suggested that the persistent infection with avian reoviruses, like that with mammalian reoviruses, is associated with mutations of viral genes (Huang et al., 1987). It is not known whether these mutations can result in antigenic variation and thus help the virus to survive immune attack.

The mechanisms of reovirus-induced tissue damage are still obscure. Reovirus-induced tenosynovitis is characterized by chronic inflammatory lesions (Chapter V). Viruses can induce tissue damage in various ways. The most straightforward mechanism is the damage of infected cells due to viral replication. However, paradoxically avian reovirus multiplies at its maximum in the intesine but does not result

in obvious enteritis. Chronic tenosynovitis or arthritis is unlikely to result directly from the cytocidal effects of the reovirus. Moreover, joint lesions start to appear when the viral titre in joint tissues starts to decline, and in fact, the lesions continue to progress even when it is very difficult to isolate any virus from affected tissues (Chapter V).

An alternative hypothesis could be that the reovirus-induced tenosynovitis is mediated by an immune-based mechanism. Persistent infection with continuous slow release of virus could lead to the formation of immune complexes which could circulate in the blood as well as being deposited in the synovium and induce a type III hypersensitivity reaction. Many viral infections of man and animals are known to be associated with formation and tissue-deposition of immune complexes (Oldstone et al.,1975). Persistently infected cells could also result in T-cell cytotoxicity or delayed (type IV) hypersensitivity, although in that case the failure to eliminate virus might be attributed to balance between virus production and cell destruction.

Because of the striking similarities in the pathology of avian viral arthritis and human rheumatoid arthritis, another hypothesis can be put forward, namely that the lesions of reovirus-induced tenosynovitis are partly autoimmune-mediated. However, rheumatoid factor has not been detected in avian viral arthritis (Walker, 1975) and these observations are

further supported by the present study (Chapter VI), although other autoantibodies, such as antinuclear antibody or anti-collagen antibody (Chapter VI), could play a similar role as that of rheumatoid factor by forming immune complexes and inducing type III hypersensitivity reaction. In addition to these suggestions, it is also possible that virus-induced injury of joint tissues could result in the release into the circulation of antigenically altered connective tissue components such as collagen (or its derivatives), which could sensitize the individual and lead to the development of type IV hypersensitivity against that tissue component. Mammalian reovirus infection in mice can lead to the development of autoimmune polyendocrine disease which is due to the presence of common antigenic determinants in the virus and the host cells (Haspel et al., 1983). A similar mechanism might operate in avian reovirus infection.

Further studies on the pathogenesis of reovirus-induced tenosynovitis should concentrate on the immunopathology of avian reovirus infection, particularly the role of reovirus-antibody complexes and autoantibodies such as antinuclear antibody and anti-collagen antibody, in the development of the disease. The following parameters merit detailed investigation:

a) possible deposition of immune complexes in the synovium,

b) activation of the complement system by the immune complexes,

c) correlation between the presence of antinuclear and anti-collagen antibodies in the sera and the development of the disease,

d) T-cell responses to reovirus persisting in joint tissues,

e) T-cell hypersensitivity to joint tissue components such as collagen, and

f) characteristics of the inflammatory cells and mediators involved in the tenosynovitis.

Finally, although reovirus-induced arthritis of chickens is not a replica of human rheumatoid arthritis, it might well serve as a valuable experimental model for chronic arthritis in other species including human rheumatoid arthritis, with particular reference to study of detailed aspects of the pathogenesis of synovial injury associated with infection and/or allergy.

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APPENDIX-A

REAGENTS, MEDIA AND BUFFERS

1. Agar gel for AGP test

Materials:

NaCl	80g or 10g (for avian or mammalian sera respectively)
Agar No. 2*	10g
1% Trypan blue	10ml
1% Merthiolate**	10ml
Distilled water	980ml

Method:

Ingredients were dissolved and sterilized simultaneously by autoclaving at 121°C for 15 minutes at a pressure of 15 pounds per sq.in., dispensed in 22 ml aliquots in universals and stored at 4°C.

When required the agar was melted in boiling water and poured into plastic petridishes at a volume of 11 ml per 60 mm dish.

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* Oxoid Ltd., London, England.

** E.I. Lilly & Co. Ltd., Basingstoke, England.

2. Antibiotic broth

This comprised nutrient broth containing 10X antibiotic.

Materials:

Nutrient broth powder*	25g
Distilled water	1000ml
Antibiotic solution (see below)	

Method:

Nutrient broth powder was dissolved in distilled water, autoclaved at 121°C for 15 minutes at a pressure of 15 pounds per sq. in., dispensed into 100 ml aliquots and stored at 4°C.

4 ml of antibiotic solution was added to 100 ml of nutrient broth immediately before use.

2.1. Antibiotic solution

Materials:

Crystapen** (1,000,000 iu benzyl penicillin sodium BP)	600mg
Streptomycin sulphate BP[a]	1g
Sterile distilled water	40ml

Method:

Penicillin and streptomycin were dissolved in distilled water. This was made fresh each time.

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* Oxoid Ltd., London, England.
** Glaxo Lab. Ltd., Greenford, England.
[a] Evans Medical Ltd., Greenford, England.

3. Bouin's solution

Materials:

Saturated picric acid	750ml
Formalin (40% formaldehyde)	250ml
Glacial acetic acid	50ml

Method:

All ingredients were added together and stored at room temperature in glass stoppered bottles.

4. Carbonate-bicarbonate buffer, 0.05M, pH 9.6

Materials:

Solution A (1M sodium bicarbonate)

Sodium bicarbonate	8.4g
Distilled water	100ml

Solution B (1M sodium carbonate)

Sodium carbonate	10.6g
Distilled water	100ml

Method:

4.53 ml of Solution A and 1.82 ml of Solution B were mixed together. Distilled water was added to make 100 ml. pH was adjusted to 9.6 by adding few more drops of either carbonate or bicarbonate solution, if required.

5. Cell culture medium M199

Materials:

Sterile distilled water	632.5ml
M199 medium (10X, with L-glutamine)*	85ml
Tryptose phosphate broth (see below)	100ml
7.5% Sodium bicarbonate*	11ml
1M HEPES buffer*	14ml
Antibiotic solution (see 2.1)	4ml
Fungizone solution (see below)	2ml

Method:

All ingredients were added together and mixed gently. The pH was between 7.2 to 7.4. Ten or five percent newborn calf serum was added to make growth or maintenence medium, respectively just before use.

5.1. Tryptose phosphate broth

Materials:

Tryptose phosphate broth powder**	29.5g
Warm distilled water	1000ml

Method:

The ingredients were mixed together, autoclaved at 121°C for 15 minutes at a pressure of 15 pounds per sq.in., dispensed into 100 ml aliquots and stored at 4°C.

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* Gibco Ltd., Paisley, Renfrewshire, Scotland.
** Oxoid Ltd., London, England.

5.2. Fungizone solution

Materials:

Fungizone (Amphotericin B)*	50mg
Distilled water	50ml

Method:

Fungizone was dissloved in sterile distilled water, divided into 2 ml aliquots and stored at -20°C.

6. DAB solution (0.05% DAB and 0.01% H_2O_2)

Materials:

Diaminobenzidine tetrahydrochloride (DAB)**

6% H_2O_2 stock solution

Method:

0.1% DAB solution was prepared by dissolving 10 mg of DAB in 10 ml of Tris-HCl buffer, pH 7.2 (see below). DAB solution was dispensed in 2 ml aliquots in aluminium foil-wraped bijou bottles and stored frozen at -20°C. Immediately before use, one aliquot was thawed quickly and mixed with equal volume of 0.02% H_2O_2 made in distilled water.

DAB was used as a substrate in immunoperoxidase staining.

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* E. R. Squibb & Sons, Inc., Princeton, New Jersy, USA.
** BDH Chemical Co. Ltd., Liverpool, England.

7. Dulbecco's phosphate buffered saline-A.

The original Dulbecco's formulation of PBS consisted of three components, A, B and C. However, components B ($CaCl_2$) and C ($MgCl_2$) were omitted from the PBS used in the present study.

Stock PBS-A

Materials:

$NaCl_2$	80.0g
KCl_2	2.0g
Na_2HPO_4	11.0g
KH_2PO_4	2.0g
Distilled water	800ml

Method:

Ingredients were dissolved in distilled water, dispensed into 80 ml aliquots, autoclaved for 10 minutes at a pressure of 10 pounds per sq.in. and stored at room temperature.

Working PBS-A

80 ml of stock PBS-A was diluted in 800 ml of sterile distilled water. 4 ml antibiotic solution (see 2.1) was added. Working PBS was stored at 4°C. This buffer was used for cell culture and other purposes requiring sterile PBS.

8. Fluorescence mountant (non fade)

Materials:

Glycerol	90ml
0.01M PBS, pH 7.5 (see below)	10ml
p-Phenylenediamine	100mg

Method:

p-Phenylenediamine was dissloved in PBS and added to glycerol. The final pH was adjusted to approximately 8.0 with carbonate-bicarbonate buffer. The solution was divided into small aliquots and stored in the dark at -20°C.

9. Formol sublimate solution

Materials:

Saturated aqueous mercuric chloride	900ml
Formalin (40% formaldehyde)	100ml

Method:

The ingredients were added together and stored at room temperature in glass stoppered bottles.

10. Karnovsky's fixative for electron microscopy

Materials:

Paraformaldehyde	2g
50% glutaraldehyde	5ml
Phosphate buffer, pH 7.4	

Method:

2 g paraformaldehyde was dissolved in 25 ml distilled water at 65°C by continuous stirring. 1 to 3 drops 1N NaOH solution was added, while stirring, to clear the preparation. After cooling the solution 5 ml of 50% glutaraldehyde and sufficient amount of phosphate buffer were added to make 50ml.

11. MTT solution

Materials:

MTT ([3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl] tetrazolium bromide)*	100mg
PBS-A	20ml

Method:

MTT was dissloved in PBS-A and sterilized by filtration through a millipore filter. The solution was divided into small aliquots and stored at -20°C.

12. Phosphate buffer, 0.01M, pH 7.0

Materials:

Na_2HPO_4 (anhydrous)	0.97g
NaH_2PO_4, $2H_2O$	0.49g
Distilled water	1000ml

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* Sigma Chemical Co. Ltd., Poole, Dorset, England.

Method:

The ingredients were dissolved in distilled water. The pH was checked and if required, adjusted with 1N HCl or 1N NaOH. This buffer was used as substrate diluent in ELISA.

13. Phosphate buffered saline (0.01M phosphate and 0.145M NaCl, pH 7.1 or 7.5)

Materials:

	pH 7.1	pH 7.5
$NaCl_2$	8.50g	8.50g
Na_2HPO_4 (anhydrous)	1.07g	1.22g
NaH_2PO_4, $2H_2O$	0.39g	0.22g
Distilled water	1000ml	1000ml

Method:

As for phosphate buffer. PBS, pH 7.1 was used in immunofluorescence and PBS, pH 7.5 was used in ELISA.

14. Tris buffered saline (0.05M Tris, 0.145M NaCl, pH 7.6)

Materials:

Tris (hydroxymethyl) aminomethane	6.1g
$NaCl_2$	8.5g
1M HCl	
Distilled water	1000ml

Method:

The ingredients were dissolved in 950 ml distilled water, the pH was brought down to 7.8 with 1M HCl and the final volume was adjusted to 1000 ml with distilled water.

This buffer was used for washing in immuno-peroxidase staining.

15. Tris-HCl buffer (0.1M Tris, pH 7.2)

Materials:

Tris (hydroxymethyl) aminomethane	12.10g
1M HCl	
Distilled water to make	1000ml

Method:

As for Tris buffered saline. This buffer was used to prepare DAB solution.

16. Tris-HCl buffer (0.05M Tris, pH 7.8)

Materials:

Tris (hydroxymethyl) aminoethane	6.10g
1M HCl	
Distilled water to make	1000ml

Method:

As for Tris buffered saline. This buffer was used in ELISA antigen preparation.

17. Tris-NaCl-EDTA (TNE) buffer (0.05M Tris, 0.01M NaCl and 0.001M EDTA, pH 7.8)

Materials:

Tris (hydroxymethyl) aminoethane	6.10g
NaCl	0.59g
EDTA (disodium)	0.37g
1M HCl	
Distilled water to make	1000ml

Method:

As for Tris buffered saline. This buffer was used in ELISA antigen preparation.

18. Trypsin solution (0.05%)

Materials:

2.5% Trypsin (1:250) solution*	10ml
PBS-A	500ml

Method:

2.5% Trypsin solution was divided into 10 ml aliquots and stored at -20°C. Immediately before use, one aliquot was thawed and diluted in 500 ml prewarmed PBS-A. This was used for disaggregation of cells during cell culture.

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* GIbco Ltd., Paisley, Renfrewshire, Scotland.

19. Veronal buffered saline (5X)

Materials:

Sodium chloride	8.50g
Sodium barbitone	0.38g
Barbitone	0.58g
Distilled water	200ml

Method:

The ingredients were dissolved in distilled water. This was a 5X concentrated solution which was diluted to single strength just before use.

APPENDIX-B

LIST OF PUBLICATIONS

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

Islam, M.R., Jones, R.C. and Kelly, D.F.(1988). Pathogenesis of experimental reovirus tenosynovitis in chickens: influence of the route of infection. Journal of Comparative Pathology,[In press].

Islam, M.R. and Jones, R.C.(1988). An enzyme-linked immunosorbent assay for measuring antibody titre against avian reovirus using a single dilution of serum. Avian Pathology,[In Press].

Islam, M.R., Jones, R.C., Al-Afaleq, A. and Kelly, D.F. (1987). Early pathogenesis of experimental reovirus infection in chickens. Paper presented in the Annual Meeting of the Association of Veterinary Teachers and Research Workers, Scarborough, 13th-15th April, 1987.

Islam, M.R.(1987). Pathogenesis of avian arthrotropic reovirus infection. Paper presented in the Faculty Forum Seminar on Arthritis, Faculty of Veterinary Science, University of Liverpool, 13th May,1987.