STUDIES ON LOCAL AND SYSTEMIC ANTIBODY RESPONSES IN CHICKEN TO AVIAN REOVIRUS INFECTIONS

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

by

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Preface

The experimental work described in this thesis was conducted in the Jordan Building, Department of Veterinary Pathology, Leahurst, University of Liverpool, U.K. between 1995 and 1997.

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Abstract

Following infection of chicks with non-attenuated avian reoviruses, the effects of age at infection, route and strain of virus on virus-specific IgA and IgG responses in the intestine and serum were investigated using anti-IgA and -IgG monoclonal antibodies in enzyme-linked immunosorbent assays. Following oral or subcutaneous infection at day old with a trypsin-resistant strain (R2), IgA was not detected in the intestinal contents, but chicks infected orally at 7 days or 3 weeks old showed a substantial rise in intestinal IgA. Following subcutaneous infection, only those infected at 3 weeks old showed an IgA response. Both routes induced similar reovirus-specific IgG but not IgA responses in the serum in all age groups. A trypsin-sensitive avian reovirus (TR1) did not replicate in the intestine after infection by either route at day old or 7 days old and no reovirus-specific IgA was detected in the intestinal contents. However, substantial antiviral IgG was elicited in sera of all TR1-infected chicks.

Hormonal and chemical bursectomy of chicks infected orally or subcutaneously with reovirus strain R2 at 7 days old caused extended virus persistence in the bursectomised / orally infected chickens compared to the intact ones. Bursectomised and orally infected chicks had a 100-fold increase in intestinal virus titre compared to the intact group. No reovirus-specific IgA was detected in the intestinal contents of any bursectomised chicks but a virus-specific IgG response in sera was delayed in all the bursectomised chicks.

An immunisation / challenge study was done in day-old specific-pathogen-free (SPF) chickens infected by eyedrop with a non-attenuated reovirus, strain R2. Virus was recovered between 3 and 7 days after the initial infection (pi) from the Harderian Gland (HG) and the gut. After challenge with R2 on day 38 pi, no virus was recovered from intestines of the immunised chicks, while all non-immunised but challenged chicks were positive. Reovirus-specific IgA and IgG were detected in tears between 7 to 21 days pi. In sera, virus-specific IgG but not IgA was detected during this time.

In a second experiment, eyedrop inoculation (ED) was compared with oral (OR) or subcutaneous (SC) routes with the same reovirus, in day-old chicks. Virus was recovered from the HG on day 3 pi in the ED-infected group only but from the gut, from 3 to 14 days pi after infection by all three routes. Reovirus-specific IgA in tears was detected between 7 and 21 days pi after all routes. However, no IgA was detected in the gut or in sera it was in negligible amounts in all groups of infection. Virus-specific IgG was detected in tears and sera after all the three infection routes without any significant difference.

Three-week old chickens were immunized by oral administration of either a trypsin-resistant avian reovirus, (R2) or a trypsin-sensitive one, (TR1). Four weeks later, when virus excretion had stopped, they were challenged with R2. After challenge, no virus was reisolated from tissues of chickens immunized with R2. However, after immunisation with TR1, challenge virus was isolated from gut and spleen and the pattern of isolation resembled that from challenged / non-immunised birds. Immediately pre-challenge, virus-specific IgA was present in equal amounts in the tears of birds immunised with both viruses, but it was in the gut of R2-immunised birds only. No virus-specific IgG was found in the gut pre-challenge after immunisation with either virus or after R2 challenge. Both TR1 and R2 induced virus specific IgA in the tears in similar amounts, but only R2 induced IgA in the gut as well as IgG in tears. In sera R2 induced substantial amounts of IgG while TR1 responded poorly. These results indicate that there is no cross-protection by TR1 to R2 infection.

Using *in vitro* intestinal explants, it was confirmed that (i) strain R2 could grow in most intestinal regions and (ii), that the anterior region of the gut actively secretes virus-specific IgA.

The trypsin-sensitive avian reovirus (TR1) was shown to be able to rapidly change to trypsin resistance in the intestine by *in vivo* passage in chicks. However, virus in the joints remained sensitive to the enzyme.

HG	Harderian gland
HI	haemagglutination inhibition
IB	Infectious bronchitis
IF	immunofluorescence
IUDR	iodo-deoxyuridine
µ	micron
Mab	monoclonal antibody
MEM	minimum essential medium
mg	milligramme
µl	microlitre
ml	millilitre
mM	milli-Molar concentration
mRNA	messenger ribonucleic acid
N	nucleocapsid protein of IB virus
ND	Newcastle disease
nm	nanometre
OD	optical density
OPD	O-phenylenediamine
p	probability that null hypothesis is valid
p.c.	post-challenge
pi.	post infection
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST80	phosphate buffered saline tween 80
PK	porcine kidney
RK	rabbit kidney

RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute media
S/C	subcutaneous
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPF	specific pathogen free
TBS	tris buffered saline
$TCID_{50}$	50% tissue culture infective dose
TKC	turkey kidney cells
TPB	Tryptose phosphate broth
Tris	(hydroxymethyl) aminomethane
Tween	polyoxyethylene sorbitan monolaurate
VN	virus neutralisation
w/v	weight/volume
YS	yolk sac

CHAPTER 1

INTRODUCTION AND AIMS OF THE THESIS

Avian reoviruses belong to the genus Reovirus within the family Reoviridae. Members of the genus are separated into two subdivisions depending on whether they are mammalian or avian in origin (Kawamura and Tsubahara, 1966). The two groups differ antigenically and further differences are that mammalian reoviruses cause haemagglutination and avian reoviruses cause cell fusion.

Avian reoviruses have world-wide distribution and are virtually ubiquitous on commercial poultry farms. They have been recovered from a variety of disease conditions in chickens and sometimes turkeys (Kibenge and Wilcox, 1983), but they are known to occur in clinically healthy flocks, where for the most part, they live a harmless existence (Mustaffa-Babjee and Spradbrow, 1971; Mustaffa-Babjee et al., 1973; Robertson et al., 1984). What conditions are necessary to induce reoviruses to cause disease, are unknown.

Disease conditions which have been associated with reovirus infection include respiratory disease, enteric disease, immunosuppression, infectious stunting syndrome and tenosynovitis / viral arthritis. The latter is the most important disease attributed to avian reoviruses (Kerr and Olson, 1967; Kibenge and Wilcox, 1983) and is a significant infectious cause of lameness, predominantly in heavy breeds, although it has been reported in commercial white leghorn laying pullets (Schwartz et al., 1976; van der Heide, 1977).

The most important features of reovirus-induced tenosynovitis which are relevant to its control are as follows. (i) Avian reoviruses can be transmitted from an infected hen to the chick,

although the proportion of infected eggs laid by an infected hen is probably low. (ii) Chicks are susceptible to infection as soon as they hatch and can easily contract infection from the environment or transovarially-infected hatch-mates via the faecal-oral route. (iii) Chickens develop resistance to infection and development of disease with age (Jones and Georgiou, 1984). (iv) Avian reoviruses are primarily enteric viruses and relicate in the gut to high titre (Jones et al., 1989). Following enteric replication, reoviruses cause disease elsewhere, especially in the joints, after virus has disseminated by viraemia. Presumably, in natural infections, if the gut has natural immunity, challenge virus will not be able to multiply and be able to cause tenosynovitis, immunosuppression, retarded growth and other effects.

There is little or no evidence to suggest that avian reoviruses are true enteropathogens (Kouwenhoven et al., 1978, 1986; Pass et al., 1982; McFerran and McNulty, 1986; Meulemans et al., 1986). In infections of chickens with another member of the family Reoviridae, rotaviruses, which are enteropathogenic, virus-specific immunoglobulin A has been found to participate both in the recovery from and resistance to infection, although it was not the sole player in protection (Myers and Schat, 1990).

In view of the high susceptibility of young chicks to reovirus infection, control measures to protect against tenosynovitis have been directed at vaccinating the young chick soon after hatch (active immunisation) or by maternally-derived antibody after breeder vaccination usually with killed vaccines.

Although a number of live and inactivated avian reovirus vaccines have been developed over some 25 years, tenosynovitis still causes losses in certain parts of the world. Failure of some vaccines may have been due to the use of poorly attenuated viruses, vaccine strains with poor immunogenicity, and presence of indigenous strains of reoviruses which were serologically different from the vaccines used.

Despite the use of reovirus vaccines for many years, little attempt has been made to understand how they work. Furthermore, the established method for testing the efficacy of vaccines is at best, convenient and at worst, irrelevant to the requirements of the vaccine. It involves application of the vaccine to very young chicks either subcutaneously or orally. Three weeks later, the chicks are challenged by footpad injection with virulent reovirus. After a few days, the feet are examined for swellings and compared with an unvaccinated challenged group. Absence of swellings in the vaccinated group indicates efficacy of the vaccine. Thus the route of challenge is unnatural and bears no relation to the real situation.

A further unsatisfactory aspect of reovirus vaccination relates to the commercial live and inactivated vaccines available. Most have been derived from the American strain S1133. While avian reovirus antigenicity is complex, and many variants exist, S1133 is probably a good choice as the basis for a vaccine from the antigenic standpoint. However, it has recently been shown that some strains of avian reoviruses are partially or completely inactivated by trypsin in the chicken intestine (Al-Afaleq and Jones, 1991; Jones et al., 1996) and S1133 and its derivative vaccines have this sensitivity. This is clearly an undesirable property for a vaccine which is needed to induce intestinal immunity (although the standard method of vaccine testing ignores the requirement for this property).

In view of the above, this thesis attempts to look at several factors relating to the immune response of the chick to avian reoviruses. The scope of the work is confined to the role played by humoral and local immunoglobulins A (IgA) and G (IgG) and it has been undertaken using two unattenuated viruses, one trypsin-resistant and another trypsin-sensitive. The immunoglobulins have been detected using an enzyme-linked immunosorbent assay and class-specific murine monoclonal antibodies. The work described should later lead on to a comparison of trypsin-resistant and -sensitive vaccines, although at present there is no trypsin-resistant product available.

The first part of the experimental work (Chapter 4) examines the production of virus-specific IgA and IgG in the intestine and sera of chicks infected orally or subcutaneously with the two viruses. The influence of age at infection was also investigated.

Previous studies on experimentally immunosuppressed chickens have indicated that recovery from infection probably involves both B- and T-cell dependant mechanisms, but that the B-cell system is likely to be protective (Kibenge at al., 1987). The role of the B-cells was only examined in terms of whole humoral antibody response. In Chapter 5, hormonal and chemical bursectomy is used to determine the role of local and systemic IgA and IgG on virus clearance in different regions of the intestine after oral or subcutaneous inoculation.

Live avian reovirus vaccines have commonly been administered by oral or subcutaneous routes but recently, a course spray method has been described (Giambrone et al., 1991, 1992). Coarse spray application will introduce much of the vaccine via the ocular route. The efficacy of application of virus by eyedrop and the possible role of the ocular lymphoid tissue and in particular, the Harderian gland, are explored in Chapter 6. A 'vaccination'-challenge experiment is described.

In Chapter 7, a further simulated 'vaccine'-challenge experiment is conducted with the intention of determining if a virus which is sensitive to trypsin and does not replicate in the intestine and therefore produces no intestinal IgA, is capable of protecting the gut against later challenge. Efficacy is assessed by the ability of the challenge virus to replicate in the gut.

Several *in vivo* studies have established that avian reoviruses replicate in the chick intestine (Kibenge et al., 1985; Jones et al., 1989) but the ability of specific regions of the gut to support virus replication has not been examined, nor the ability of the different regions to produce local virus-specific antibodies. In Chapter 8, explanted pieces of different regions of the intestine are cultured

and tested for virus susceptibility. In addition, intestinal explants taken from previously infected chicks are tested for their ability to produce virus-specific IgA and IgG.

Recent work (Mosos, 1994; Jones et al., 1996) has shown that passage of trypsin-sensitive reoviruses through the chick after infection at high dose can cause the virus to become resistant to the enzyme. This is explored on Chapter 9 with regard to route of infection and nature of the virus persisting in different tissues.

Chapter 10 draws together the main conclusions from the experimental work and attempts to point to a direction in which future work should take.

CHAPTER 2

A REVIEW OF THE LITERATURE ON AVIAN REOVIRUSES

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CHAPTER 2

A REVIEW OF THE LITERATURE ON AVIAN REOVIRUSES

2.1. A history of reoviruses

2.1.1. The family Reoviridae

The term "REOVIRUS" was suggested by Sabin (1959) to define a group of medium to large viruses which had earlier been classified as type 10 ECHO (enteric cytopathic human orphan) viruses. These REOviruses (respiratory and enteric orphan viruses) were isolated from the alimentary and respiratory tracts of humans, of which many were apparently healthy (Robbins et al., 1951; Ramos-Alvarez and Sabin, 1954).

There are now six genera in the family Reoviridae. These are Reovirus (Orthoreovirus), Orbivirus, Rotavirus, Cypovirus, Phytovirus and Fijivirus (Joklik et al., 1983). Members of the Reovirus, Orbivirus and Rotavirus genera infect man and animals. Members of the the Fijivirus and Phylovirus genera infect plants, while those in the Cypovirus genus affect insects.

The main morphological characteristics of the family Reoviridae are size, which is approximately 56 to 82 nm and the icosahedral symmetry. The virions are non-enveloped with a double protein capsid shell. These viruses have a segmented genome with double stranded RNA (dsRNA). The genome consists of 10-12 segments. Virus replication is fully cytoplasmic.

2.1.2. The genus Reovirus

The genus Reovirus consists of three groups of viruses: mammalian and avian reoviruses and the unique Nelson Bay virus (Joklik, 1974). Avian and mammalian reoviruses share morphological and physicochemical properties (Petek et al., 1967). However they can be differentiated by host range, lack of common antigenicity and lack of haemagglutination by the avian reoviruses. They can further be differentiated since the cytopathic effect (CPE) of avian reoviruses in cell cultures is cell fusion with formation of synctia (van der Heide, 1977; Olson, 1978) which does not occur with mammalian reoviruses (Kawamura et al., 1965; Gomatos and Tamm, 1963).

The Nelson bay virus was isolated from a flying fox *Pteropus poliocephalus* (Gard and Compans, 1970) and is unique in that it has characteristics that place it intermediate between mammalian and avian reoviruses. It has common complement fixing and immunofluorescence antigens with mammalian reoviruses but possesses the cell fusion characterisics of avian reoviruses (Gard and Compans, 1970; Gard and Marshall, 1973).

2.1.3. Mammalian reoviruses

All mammalian reoviruses share a common antigen which is detectable by complement fixation and agar gel precipitation tests (Sabin, 1959; Leers et al., 1989). Mammalian reoviruses are divided into three different but related serotypes (1, 2 and 3) by means of serum neutralisation and haemagglutination inhibition tests (Sabin, 1959; Rosen, 1960; Behbehani et al., 1966). These viruses have been reported from a wide range of mammmals and reptiles (Joklik, 1974). Although all mammals may be susceptible to infection with these viruses (Spendlove, 1978), the relationship between mammalian reoviruses and disease conditions is not well defined (Tyler and Fields, 1986).

2.1.4. Avian reoviruses

The first isolation of an agent later to be identified as an avian reovirus was described by Fahey and Crawley (1954) from an outbreak of chronic respiratory disease in chickens. In the same year, another infectious agent was isolated from cases of tenosynovitis in chickens (Olson et al., 1954). These agents were found to be able to cause arthritis after experimental infection (Olson et al., 1956). The agents were characterised as reoviruses by Petek et al. (1967). In 1966 an agent which had been suspected of being a mycoplasma was later characterised as a virus by Olson and Kerr (1966) and Taylor et al. (1966). This was found to be an avian reovirus by Walker et al. (1972).

Subsequent to the identification of avian reovirus as a pathogen for chickens, many isolations of reovirus in disease conditions were reported from different parts of the world (Kibenge and Wilcox, 1983; Robertson and Wilcox, 1986). A similar virus was isolated in the UK from mature broiler breeders suffering from arthritis and tenosynovitis with rupture of the gastrocnemius tendon (Jones et al., 1975).

2.2. Physicochemical properties of avian reoviruses

2.2.1. Density

The density of intact infectious virions of avian reoviruses was found to be between 1.36 and 1.37 g/ml, using caesium chloride gradients (Glass et al., 1973; Schnitzer et al., 1982). The non-infectious virus particles band has a gradient from 1.29 to 1.30 g/ml (Spandidos and Graham 1976; Schnitzer et al., 1982). Viral core particles which are non-infectious have a density of 1.44 g/ml (Schnitzer et al., 1982). Similar values have been found with the mammalian reoviruses (Joklik, 1974).

2.2.2. Sensitivity to lipids and other chemicals

Avian reovirus was found resistant to 2% formaldehyde at 4^0C (Meulemans and Halen, 1982). The Fahey-Crawley virus was partially inactivated by 2% phenol after 24 hours at room temperature and was completely inactivated by 100% ethyl alcohol (Petek et al., 1967).

The virus is resistant to ether (Glass et al., 1973; Dutta and Pomeroy, 1967b) and either partially or completely resistant to chloroform (van der Heide and Kalbac, 1975; Glass et al., 1973; Carboni et al., 1975; Jones et al., 1975). Concentrations of phenol and mercury bichloride non-toxic to chick embryos were found to inactivate avian reoviruses (Dutta and Pomeroy, 1967b; Deshmukh and Pomeroy, 1969b).

2.2.3. Sensitivity to physical and chemical treatment

Avian reoviruses are stable over a wide pH range. The virus was resistant to pH 3.0 and pH 9.0 for four hours (Glass et al., 1973). The virus was also found resistant to pH 3.0 for 30 minutes (Jones et al., 1975; van der Heide and Kalbac, 1975; Mustaffa-Babjee et al., 1973) and to pH 3.0 for 24 hours at 40^0C (Kawamura et al., 1965). Carboni et al. (1975), using a similar procedure to that of Kawamura et al. (1965), found avian reoviruses partly sensitive to pH 3.0 and pH 12 but stable at pH 5.

Ambient temperatures favour the survival of avian reoviruses. The virus is stable at 50^0C for up to two hours (Kawamura et al., 1965; Petek et al., 1969; Deshmukh and Pomeroy, 1969b; Rossi et al., 1969; Carboni et al., 1975; Levisohn and Weisman, 1980). Partial inactivation of isolate RAM-1 at 56^0C for 30 minutes and the Fahey-Crawley virus for 1 hour has been documented (Mustaffa-Babjee et al., 1973; Petek et al., 1967). Isolate S-1133 was shown to be completely inactivated at 55^0C in less than an hour (Spadidos and Graham, 1976). At lower temperatures, avian

reoviruses are stable and survive for long periods (Robertson and Wilcox, 1983).

Earlier work had shown avian reoviruses to be resistant to proteolytic enzymes, especially trypsin (Kawamura et al., 1965; Petek et al., 1967; Jones et al., 1975). Later, Gouvea and Schnitzer (1982b) showed possible inactivation of some strains by the effect of gastrointestinal enzymes. Al-Afaleq and Jones (1991) confirmed that an avian reovirus TR1 originally isolated from a turkey was trypsin-sensitive and others have been shown to have this property (Jones et al., 1996).

2.2.4. Haemagglutination

Avian reoviruses have been found to lack the ability to agglutinate erythrocytes of the chicken, duck, goose, turkey, human type O, cattle, guinea pig, mouse, rabbit, rat or sheep (Taylor et al., 1966; Deshmukh and Pomeroy, 1969a; Deshmukh et al., 1969; Glass et al., 1973; Mustaffa-Babjee and Spradbrow, 1973; Levisohn and Weisman, 1980; Hieronymus et al., 1983a and b). However, there are two reports which claim haemagglutinating activity for some avian reoviruses (Dutta and Pomeroy, 1967b; Gershowitz and Woolley, 1973), but in general, it is accepted that they do not haemagglutinate.

2.2.5. Molecular properties of avian reoviruses

The avian reovirus virion consists of an outer capsid and an inner structural layer, with both double-stranded ribonucleic acid (dsRNA) and single stranded (ssRNA) genome (Koide et al., 1968; Sekiguchi et al., 1968; Spandidos and Graham, 1970). The compositions have been calculated and the mean percentages are 18.7% RNA and 81.3% protein (Sekiguchi et al., 1968).

The RNA content of avian reovirus is higher than that of mammalian reovirus which has been reported to be 13%-15% (Gomatos and Tamm, 1963; Mayor et al., 1965). The difference in

RNA content of the two reovirus groups is attributed to the larger amount of ssRNA contained in avian reovirus (Koide, 1970).

2.2.6. Avian reovirus genome

Evidence for the presence of RNA in avian reovirus is that virus replication is not inhibited by the DNA inhibitors IUDR or BUDR (Kawamura et al., 1965; Deshmukh and Pomeroy, 1969b; Mustaffa-Babjee et al., 1973; Carboni et al., 1975; Jones et al., 1975; Hieronymus et al., 1983a) and that virus is resistant to DNase but sensitive to RNase at low ionic concetrations (Deshmukh and Pomeroy, 1969b; Spadidos and Graham, 1976).

Like that of the mammalian reovirus, the genome of avian reovirus consists of dsRNA. Evidence for the double-stranded nature of the viral genome is provided by the following: (i) infected cultures fluorescence pale green when treated with acridine orange (Glass et al., 1973); (ii) the RNA is not affected by treatment with formaldehyde (Sekiguchi et al., 1968); (iii) the RNA exhibits very sharp melting profiles, with the melting point depending on the ionic strength (Koide et al., 1968; Spandidos and Graham 1976); (iv) the RNA is resistant to RNAse treatment, the resistance depending on monovalent and divalent cationic concentrations (Spandidos and Graham, 1976); and (v) the RNA base composition indicates equality of adenine and uridine, as well as guanine and cystosine (Koide et al., 1968; Sekiguchi et al., 1968; Spandidos and Graham, 1976).

The avian reovirus genome is known to be segmented and the segments are separated into three size classes which sediment at 14, 12, and 11S in sucrose gradients (Spandidos and Graham, 1976). By use of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), each of these three segments have been further separated into size classes which contain a total of 10 discrete molecular species (Spandidos and Graham, 1976; Gouvea and Schnitzer, 1982a, b).

The three sizes of segments are (i) the three large (L1, L2 and L3) (ii) three medium (M1, M2 and M3) and (iii) four small (S1, S2, S3 and S4). The large segments have molecular weights of 2.4-2.7x10^6, the medium size 1.3-1.7x10^6, and the small sizes 0.68-1.2x10^6.

Although the genomic dsRNA segmentation of avian reoviruses was shown to be similar to that of the mammalian (Joklik, 1981), there was some marked difference in the SDS-PAGE rate of migration of the S1 segment. This segment had a higher molecular weight in avian reoviruses than in its mammalian counterpart and it therefore migrated more slowly. The segment S1 of the mammalian reovirus codes for two proteins (Jacobs and Samuel, 1985) whereas the larger S1 gene of avian reovirus appears to code for only one low molecular weight protein (Schnitzer, 1985).

Analysis of a number of field isolates of avian reoviruses with SDS-PAGE has established marked polymorphism of migration patterns of dsRNA segments, first among isolates of the same serotypes and secondly even between different serotypes (Gouvea and Schnitzer, 1982b). The polymorphism in migration shown by the mammalian reoviruses is characteristically similar and that shown by the avian reoviruses is also similar (Gouvea and Schnitzer, 1982b). This heterogeneity in the migration profile gives apparently no correlation between electrophenotype and antigenicity or disease status (Gouvea and Schnitzer, 1982b; Robertson and Wilcox, 1986; Rekik et al., 1990; Clark et al., 1990; Lozano et al., 1992).

2.2.7. Proteins of avian reovirus

Complete avian reovirus particles are composed of proteins that can be separated by SDS-PAGE into the same three classes as reported with the mammalian reoviruses (Joklik, 1981), namely the large, medium, and small (Nick et al., 1975; Spandidos and Graham, 1976; Schnitzer et al., 1980).

Only two proteins have been identified in avian reoviruses, while three proteins (1, 1c and 2) are present in mammalian reoviruses (Joklik, 1981). The proteins 1 and 2 are situated in the core of the mammalian viruses, and 1c, a cleavage product of 1, is the only protein present on the surface of the virion and it is regarded as the major component of the 12 core spikes (White and Zweerink, 1976). In avian reoviruses, two proteins (A and B) have been demonstrated on the surface of the virus particles (Schnitzer et al., 1982). The TC protein present in the top component of avian reovirus is not found in infectious virions (Schnitzer et al., 1982), nor has it been reported in mammalian reovirus top component particles (Smith et al., 1969).

2.3. Cultivation of avian reoviruses

2.3.1. In embryonated eggs

Avian reoviruses have been successfully grown in embryonated eggs following inoculation by both the the yolk sac (YS) route and the chorioallantoic membrane (CAM) routes, but with variable results using the allantoic cavity (AC). Inoculations by the YS route into embryonated eggs of 5 - 6 day of incubation produced 100% mortality in 72 to 96 hours and the embryos were slightly dwarfed and purplish, due to severe subcutaneous hemorrhages (Olson et al., 1954; Petek et al., 1954; Glass et al., 1973). This route was also used for virus isolation (Olson et al., 1954; Fahey and Crawley, 1954; Glass et al., 1973) and virus titration (Petek et al., 1967; Olson and Kerr, 1970; Olson and Weiss, 1972; Guneratne et al., 1982).

Inoculation of embryonated eggs of 9 to 10 days incubation by the AC has been used for avian reovirus isolation (Fahey and Crawley, 1954; Glass et al., 1973; Guneratne et al., 1982). Virus propagation and titration have also been done by this route (Bains et al., 1974; Petek et al., 1967; Olson and Weiss, 1972). McFerran et al. (1971) and Deshmukh and Pomeroy (1969c) have, however reported failures of virus isolation using the allantoic cavity route.

Of the three inoculation routes, the CAM method in 9 to 11 day old eggs has been the most successful for avian reovirus isolation and propagation (Dutta and Pomeroy, 1967a; Deshmukh and Pomeroy, 1969a; Johnson, 1972; Bains et al., 1974; Spradbrow and Bains, 1974; van der Heide et al., 1974; Carboni et al., 1975; Schwartz et al., 1976; MacKenzie and Bains, 1977). Viral activity was indicated by embryo mortality after 3 to 5 days, with large plaques on the thickened CAM which later became oedematous and necrotic, while surviving embryos ended up stunted with enlarged discoloured livers (Deshmukh and Pomeroy, 1969c; Bains et al., 1974; Olson, 1975; Mustaffa-Babjee et al., 1973; Spradbrow and Bains, 1974; Guneratne et al., 1982).

2.3.2. Cell culture

The successful growth of avian reoviruses and their characteristic cytopathic effect (CPE) has made cell-cultures a popular method of virus isolation and propagation. Cell cultures used so far have included chick embryo fibroblasts (CEF) (Lee et al., 1973; Mustaffa-Babjee et al., 1973; Bains et al., 1974; Spradbrow and Bains, 1974; van der Heide and Kalbac, 1975), chick embryo lung (CELu) (Petek et al., 1967; Jones et al., 1975; Sahu and Olson, 1975; Bradbury and Garuti, 1978; Hussain et al., 1981), chick embryo liver (CELi) (McFerran et al., 1976a; Jones et al., 1981; Gouvea and Schnitzer, 1982) and chick embryo kidney (CEK) (Glass et al., 1973; Bagust and Westbury, 1975; Hieronymus et al., 1983a and b).

Chicken kidney cells from birds varying in age from 1 to 3 months old have been used for virus isolation and propagation (Kawamura et al., 1965; Kaji et al., 1970; McFerran et al., 1971; Menendez et al., 1975; Sahu and Olson, 1975; Green et al., 1976) as well as mature chicken testicular cells (Sahu and Olson, 1975). Fujisaki et al. (1967) titrated a turkey avian reovirus in turkey kidney cells (TKC) obtained from 1 to 5 days old poults.

The characteristic cytopathic effect (CPE) in cell-cultures infected with avian reoviruses is the formation of multinucleated giant cells (syncytia). Ultimately, the monolayer is destroyed and bits of it float in the fluid. Staining of infected cultures with haematoxylin and eosin shows in the beginning eosinophilic but later basophilic intracytoplasmic inclusion bodies (Kawamura et al., 1965; Petek et al., 1967; Mustaffa-Babjee et al. 1973; Guneratne et al., 1982).

In an extensive study using a total of 22 reoviruses, Guneratne et al. (1982) compared the cultivation of avian reoviruses in primary cell cultures of CEF, CELi, CELu, CEK and CK and embryonated chicken eggs inoculated via the YS, CAM and AC routes. It was found that for primary isolation of avian reovirus from field specimens, CELi monolayers were more sensitive than eggs inoculated by any route and any of the other cell cultures compared. Specific advantages of the CELi were (i) plaques appeared much earlier than in other cell types (ii) higher titres of virus were produced, (iii) they required a shorter adsoption time and (iv) produced better-defined syncytia than CK cells.

Successful adaptation of avian reovirus strain WVU-2937 to mammalian cell line was made by Barta et al. (1984) in African green monkey kidney (Vero), Crandell feline kidney (CRFK), Georgia bovine kidney (GBK), baby hamster kidney (BHK), rabbit kidney (RK) and porcine kidney (PK) cells.

Haffer et al. (1984) described the use of blood monocytes-derived macrophages to cultivate avian reovirus, which resulted in production of rounded cells and detachment from the vessel surface after infection. Rabbit bone marrow cells could not support viral replication (Barta et al. 1984) but chicken bone-marrow-derived macrophages did (Bulow and Klasen, 1983).

Although, Mills and Wilcox (1993) reported that four antigenic types of Australian avian reovirus failed to replicate in lymphocytes from the bursa and thymus, Shapouri et al. (1994) did show the ability of chicken lymphoblastoid cell lines to support avian reovirus growth.

2.4. Antigenicity of avian reoviruses

2.4.1. Group specificity

Mammalian reoviruses have been grouped into three serotypes according to their behaviour in haemagglutination and virus-neutralization tests (Rosen, 1960; Leers et al., 1968; Sabin, 1959; Joklik, 1981) but they shared the compliment fixation and agar-gel-precipitation antigens (AGP) (Joklik, 1974). Mammalian reovirus are antigenically unrelated to avian reovirus (Kawamura and Tsubahara, 1966).

Avian reoviruses have shown group-specific antigens in serological tests, by use of (AGP), immunofluorescence (IF) and enzyme-linked immunosorbent assay (ELISA). AGP has been used to screen for serological responses after infection and to identify viral isolates as avian reoviruses (Kawamura and Tsubahara, 1960; Deshmukh et al., 1969; Olson and Weiss, 1972; Glass et al. 1973; van der Heide et al., 1974; Sahu and Olson, 1975; Ide and DeWitt, 1979; Wood et al., 1980; Mukit et al., 1988; Wei et al., 1988; Chauhan et al., 1988), while IF staining has been used to demonstrate group-specific antigens in the cytoplasm of infected tissue cells (Olson, 1978). Strong cross-reactions, between five Japanese avian reovirus prototype strains, indicating group-specificity, were observed using a direct IF test (Kawamura and Tsubahara, 1966).

The direct IF test has been used to demonstrate viral cytoplasmic inclusions and distribution of avian reovirus of infected chickens

(Kawamura and Tsubahara, 1966; Menendez et al., 1975b; Jones and Onunkwo, 1978) while the indirect IF has also been used in serological surveys to detect antibodies (Ide, 1982).

Using an ELISA, Slaght et al. (1978) showed that antisera to avian reoviruses S-1133, UMI-203, Reo 25 and Winterfield prepared in chickens, cross-reacted with viral antigens prepared from strain S-1133, indicating that the test showed group-specificity.

2.4.2. Type specificity

The type-specific antigens of avian reoviruses have been shown by their heterogeneity in serum neutralisation tests using different strains isolated worldwide. Seventy-seven Japanese avian reovirus isolates were classified into five serotypes by plaque reduction tests in CK cells (Kawamura et al., 1965; Kawamura and Tsubahara, 1966). The WVU-2937 strain was described as belonging to the same antigenic group as the Fahey-Crawley, virus based on one-way serum neutralisation tests on CAMs of embryonated eggs (Olson and Weiss, 1972). It was however later demonstrated using neutralisation kinetics that the Fahey-Crawley and the WVU-2937 were antigenically distinct (Munro and Woolley, 1973), a fact which was confirmed when nine isolates from USA were classified into four serotypes on the basis of cross-neutralisation tests using a constant virus-varying serum plaque reduction test in CK cells (Sahu and Olson, 1975). There was also significant cross-neutralisations among these virus isolates and two of them, WVU-2986 and WVU-1675 could not be assigned to specific serotypes.

Sahu et al. (1979) reported the classification of four avian reoviruses (Texas, UMI-203, WVU-2939 and S-1133) isolated from synovial fluid and breast blisters of chickens as subtypes of the same serotype on the basis of plaque reduction tests in CK cells cultures.

Wood et al. (1980) compared the reovirus strain S-1133 to five other reoviruses from the UK, Germany and the USA using a plaque reduction test in CK cells and grouped the six into three serotypes. The three prototypes groups were then compared with the five Japanese serotypes of Kawamura et al. (1965) and the four USA serotypes of Sahu and Olson (1975). The antigenic distinctness of the Japanese and USA serotypes were confirmed, but in contrast to Sahu et al. (1975), Wood et al. (1980) classified WVU-2937 and S-1133 as separate serotypes. As a result of this, at least eleven avian reovirus serotypes were confirmed, although considerable cross-neutralisation between heterologous types was observed and this has made it difficult to assign some strains to discrete groups (Robertson and Wilcox, 1984).

Avian reovirus heterogenicity of antigens in neutralisation test has been confirmed (Kawamura et al., 1965; Deshmukh and Pomeroy, 1969a; Olson and Weiss, 1972; Sahu et al., 1979; Wood et al., 1980; Hieronymus et al., 1983; Robertson and Wilcox, 1984) and the presence of more than one protein involvement in the neutralisation process is suggested as the cause (Robertson and Wilcox, 1984).

2.5. Pathogenesis and epizootiology

Avian reoviruses have a worldwide occurrence and are ubiquitous. Although they have been found in a number of avian species, domestic chickens and perhaps turkeys are the only recognized natural or experimental hosts for reovirus-induced arthritis/tenosynovitis.

The occurrence of reoviruses in other avian species including the turkey (van der Heide et al., 1980; Nersessian et al., 1986), ducks and zoo wedge-tailed eagle (*Aquilla andax*) (Jones and Guneratne, 1984) may raise the issue of cross-infection between species.

Strains isolated from turkeys have been found to be pathogenic for the chicken but not *vice versa* (Al-Afaleq and Jones, 1989).

A number of factors have been considered to influence the outcome of reovirus infection (Kibenge and Wilcox, 1983). Those related to the virus include the virulence of the strain (Gouvea and Schnitzer, 1982; Jones and Guneratne, 1984), the dose (Gouvea and Schnitzer, 1982), and the route of infection (Kibenge and Wilcox, 1983; Islam et al., 1988).

Those related to the host include the breed, heavy breeds being more susceptible than light ones (Schwartz et al., 1976; van der Heide, 1977; Jones and Kibenge, 1984), and the age at infection, younger birds being more susceptible than older ones (Jones and Georgiou, 1984; Rosenberger, 1983; Montgomery et al., 1986; Roessler and Rosenberger, 1989).

2.5.1. Pathogenesis

Following experimental infection of adult SPF hens via the nasal, tracheal or oesophageal routes, reovirus was recovered from all areas of the respiratory, enteric and reproductive tracts and the tendons of the hock joint (Menendez et al., 1975). In a related study, Kibenge et al. (1985) orally infected day-old SPF chicks with R2 strain of reovirus, and isolated virus from the plasma, erythrocyte and mononuclear fractions of the blood within 30 hours pi and by 3 to 5 days virus had been distributed thoughout the body. Although these reports are indicative of widespread virus dissemination, as a result of viraemia, the principal site of virus replication is the enteric tract (Kibenge et al., 1985) and virus finally settles in the hock joints and tendons where it usually persists (Jones and Kibenge, 1984; Jones and Guneratne, 1984; Jones and Onunkwo, 1978; Sahu and Olson, 1975; Jones et al., 1989).

Strain differences in the pathogenicity of reoviruses have been reported (Gouvea and Schnitzer, 1982; Rosenberger, 1983; Jones and Guneratne, 1984; Kibenge and Dhillon, 1987; Tang et al., 1987; Rosenberger et al., 1989; Roessler and Rosenberger, 1989; Clark et al., 1990; Ni and Kemp, 1995). Furthermore, trypsin-sensitive strains of avian reoviruses have recently been described (Al-Afaleq and Jones, 1991; Jones et al., 1996) which have varied pathogenicity in the gut.

2.5.2. Transmission

Reovirus infections can spread easily through large groups of birds and under commercial conditions the virus can be isolated from faeces of chicken between the second and fourth weeks of life (McFerran and McNulty, 1993).

*2.5.2.1 **Horizontal transmission*** Lateral transmission of virus occurs and is considered to be the most important route of spread (Kerr and Olson, 1969; Olson and Khan, 1972; van der Heide et al., 1975; Jones and Onunkwo, 1978; MacDonald et al., 1978; Jones and Guneratne, 1984). Spread occurs largely by ingestion of infected faecal material, contaminated feed and water, and is fairly rapid, as shown by early virus recoveries after two weeks, and positive agar-gel precipitin reactions after three weeks in in-contact chickens (Jones and Onunkwo, 1978; Jones and Guneratne, 1984). The principal route of excretion of virus from an infected bird is via the intestinal tract (Jones et al., 1975). Virus was shown to be short-lived in the trachea (Jones and Onunkwo, 1978), which implied that spread by the respiratory route may be limited.

Because avian reoviruses are relatively resistant to heat and disinfectants, it is likely that virus is capable of surviving in contaminated hatcheries and poultry houses between batches of chicken and well as in the environment (Robertson and Wilcox, 1986), unless thorough cleaning and disinfection are carried out.

2.5.2.2. Vertical transmission This form of spread was first suggested by Deshmukh and Pomeroy (1969c), when they reported the isolation of reovirus from chicks from naturally infected breeders. Later, this was supported by recovery of reovirus from chickens derived from infected hens (Glass et al., 1973) but in both this and the previous report, the possibility of post-hatch exposure to avian reovirus as source of infection was not eliminated. Natural infection of embryos was first confirmed by the isolation of reoviruses from cell cultures prepared from apparently normal embryos (Mustaffa-Babjee and Spradbrow, 1971; Mustaffa-Babjee et al., 1973; Chubb and Ma, 1974) and later supported by Yamada et al. (1977), who isolated avian reovirus from dead-in-shell embryos. In a simulated egg transmission trial conducted by Jones and El-Taher (1985), 7-day incubated SPF eggs were inoculated via the yolk sac with graded doses of avian reovirus R2 and virus was recovered from the liver and hock joints of the hatched chicks at 7 days of age.

Avian reoviruses were conclusively shown to be vertically transmitted by Menendez et al. (1975a) when they experimentally infected SPF white leghorns breeders with avian reovirus and were able to recover virus from the progeny, although at a low rate. Recent work which further showed vertical transmission was reported by Vielitz et al. (1989) and Giambrone et al.(1991).

The susceptibility of avian reoviruses to trypsin and other enzymes in the intestinal tract may be one of the factors which are likely to affect vertical-transmission. Following experimental infection, egg-transmission of a trypsin-sensitive (TR1), avian reovirus was found to occur much less frequently than a trypsin-resistant strain (R2) (Mufarrej et al., 1996).

Although vertical transmission of avian reoviruses virus usually occurs at a low rate, it is an important method of spead because

it can cause a focus of infection for eventual horizontal spread to in-contacts (McFerran and McNulty,1993).

2.5.2.3 Infection through the skin Another possible form of transmission was demonstrated by Al-Afaleq and Jones (1990) when they showed that broken skin of the footpad could act as a route of entry from virus-infected litter, after which virus settled in the hock joint. This form of transmission is likely to be of importance in situations where there is poor straw, where birds are likely to fight, and where the skin is made weak due to nutritional ailments.

2.5.3. Persistence

After inoculation of chickens, avian reoviruses replicate in the intestines (Glass et al., 1973; MacDonald et al., 1978; Menendez et al., 1975b), and following a viraemia, later may settle in the hock joints. Persistence of reovirus in the joints has been shown for at least 13 weeks (Jones and Onunkwo, 1978) and as long as 285 days p.i. (almost 41 weeks) (Kerr and Olson, 1969). The relative difficulty and occasional failure to isolate virus from the joints of experimentally or naturally infected birds (Jones et al,. 1981), may indicate a strategic mechanism for viral persistence. Whether the hock joint provides the reovirus with an immunologically privileged situation is not known.

In an attempt to elucidate the mechanism of avian reovirus persistence, Huang et al. (1987) demonstrated that two genes, S4 and S2 segments, underwent some degree of mutation and these changes were associated with persistent infection. The genetic polymorphism of avian reoviruses (Gouvea et al., 1972) may also contribute to changes in the genome of the virus either due to high mutation rate or to genetic reassortment (Ramig and Fields, 1983).

Another factor which may be relevant to avian reovirus persistence is the sensitivity of some strains to digestive enzymes especially trypsin. Viral persistence in the intestine, liver, heart and hock joint tissues have been reported to occur only in chickens inoculated with trypsin-resistant strains of reovirus (Al-Afaleq and Jones, 1991; Drastini et al., 1994).

2.5.4. Clinical signs of disease

Lameness with swollen hock joints in birds usually more than five weeks of age are the main clinical signs associated with avian reovirus infection (Jordan, 1990). "Tenosynovitis" was the term used to describe the pathological changes observed in the tendon sheaths and tendons of broilers, a condition which was found distinct from infectious synovitis due to *Mycoplasma synoviae* (MS) (Dalton and Henry, 1967). This differentiation of tenosynovitis from infectious synovitis was confirmed by Olson and Solomon (1968) when they reported a natural outbreak of tenosynovitis in MS-negative broiler chickens.

In acute reovirus infections, apart from lameness, some chickens are stunted and in chronic infections the lameness is more pronounced, with swollen hock joints. The mortality is usually low (1-10%) (Kibenge and Wilcox, 1983), while the morbidity is very variable (Jordan and Pattison 1996). Because the clinical signs may resemble those caused by other infectious agents, especially mycoplasmas and staphylococci, for diagnosis, it is necessary to demonstrate the presence of virus by laboratory methods (Kibenge and Wilcox, 1983).

2.5.5. Gross lesions

In acute cases, the gross lesions of reovirus-induced arthritis consist of swelling and inflammation of the digital flexor and metatarsal extensor tendons, the hock joint and tendon sheaths just above the joint and along the posterior aspect of the shanks (McFerran and McNulty, 1993). Thickening of tendons with excess serous fluid in the joint has been described (Jones et al., 1975; van der Heide, 1977; MacDonald et al. 1978). Affected tendons become firm and fibrotic and in more advanced cases there are adhesions between the tendon sheaths and skin, causing immobility in the tendon (Johnson and van der Heide, 1971; MacDonald et al., 1978).

Jones and Onunkwo (1978) described cases where digital flexor tendons immediately below the hock joint showed hard uneven swellings of up to 2 to 3 times their normal width. In other instances the swellings within the tendon sheaths were found to be due to ruptured digital flexor tendons folding back on themselves. Rupture of the gastrocnemius tendon which takes place most frequently at the level of the hock joint has been found in very severe cases of tenosynovitis (Jones et al., 1975). Some haemorrhages and oedema deep in the tendons, followed by fibrosis and cartilage formation have been described by Jones et al. (1975). Erosion and pitting on the articular surface of the distal tibia have frequently been observed in the hock joints (Jones and Onunkwo, 1978; McFerran and McNulty, 1993).

2.5.6. Microscopic lesions

Thickening of the tendons and sheaths is caused by oedema, hyperplasia of the synoviocytes, villous proliferation of the synovium and inflammatory cell infiltration (Kibenge and Wilcox, 1983). As the disease progresses, the loose connective tissue occasionally infiltrates into the tendon, adhering to the tendon sheaths (Kibenge and Wilcox, 1983; McFerran and McNulty, 1993). Depending on the pathogenicity of the reovirus involved, some of the microscopic inflammatory changes in the tendons may occur without development of gross lesions (Olson and Khan, 1972).

Sometimes, histological changes similar to those described in viral tenosynovitis occur in synovitis caused by *Stapylococcus aureus* and the differences are only a matter of degree (Kibenge and Wilcox, 1983; Tang et al., 1987). However, a more detailed examination showed that histological changes caused by reovirus were characterized by diffuse lymphocytic inflammation of the tendon, peritendineum, and synovium, whereas those caused by staphylococci were of a focal purulent synovitis (Hill et al., 1989).

Microscopic lesions in other tissues reported in association with natural or experimental tenosynovitis virus infection have been described in the liver, spleen and bursa (Hieronymus et al., 1983; Hill et al., 1989; Roessler and Rosenberger, 1989; Tang et al., 1987; Kerr and Olson, 1969). Pericarditis and myocarditis have been consistently reported by a number of workers to the extent that they were suggested as diagnostic signs for viral arthritis in the presence of tenosynovitis (Kerr and Olson, 1969; Glass et al., 1973; Hieronymus et al., 1983; Tang et al., 1987). Histological changes have also been seen in some parts of the nervous system such as the ischiatic nerves (Itakura et al., 1977) and the brain (Kerr and Olson, 1969) but their significance is not known.

2.6. Immune response to avian reoviruses

The immune response to viral infections involves important mechanisms by which the host resolves infection and protects itself from re-infection by the same virus.

2.6.1. Humoral immunity

The humoral immune responses of the chicken to infection by avian reovirus have been detected and evaluated by a number of serological methods, usually for practical purposes, i.e. for monitoring infection or vaccine uptake. These include the agar-gel precipitation (AGP) test (Olson and Weiss, 1972; van der Heide et al., 1974), virus neutralization by use of reduction of plaque

formation in cell cultures (Kawamura et al., 1965) or on the CAM of chicken embryos (Deshmukh and Pomeroy, 1969a), virus micro-neutralization on the basis of inhibition of cytopathic effects (CPE) in cell cultures (Rau et al., 1980; Jones and Kibenge, 1984), indirect immunofluorescence (IF) (Adair, 1987; Ide and De Witt, 1979) and enzyme-linked immunoassay (ELISA) (Slaght et al., 1978, Islam and Jones, 1988; Giambrone and Solano, 1988).

AGP, IF and ELISA detect group-specific antibodies, while virus neutralisation detects type-specific antibodies (McFerran and McNulty, 1993).

Precipitating and neutralizing antibodies have usually developed in serum 2 to 3 weeks after infection (van der Heide, 1974; Jones and Onunkwo, 1978; Mukiibi-Muka et al., 1984). The development of these antibodies is either enhanced or delayed depending on a number of factors like age, breed, strain of virus and route of inoculation. AGP antibodies have been suggested to develop more rapidly in chickens of 2 weeks or older than in younger ones (Kaji et al., 1970; Jones and Georgiou, 1984). Jones and Kibenge (1984) reported that virus-neutralizing antibodies appeared earlier (at 3 weeks) in lighter breeds than in broilers (4 weeks) after infection. The proportion of birds showing positive AGP reactions is reported to be higher after infection by parental routes than after oral infection (van der Heide et al., 1974; Jones and Georgiou, 1984), although other workers have failed to find any significant difference in the immune response following different routes of infection (Islam et al., 1988).

Despite the widespread use of these tests for monitoring infection and vaccine uptake over many years, only one study has been undertaken to correlate the level of antibodies elicited with protection. Takase et al. (1996) showed that actively immunised birds with mean neutralising antibody titres of 1:238 or higher showed good protection (71%) against footpad challenge with the homologous virus. How these values relate to ELISA antibodies is not known.

2.6.2. Local immunity

Local immunity to avian reovirus infection has been paid almost no attention. The only study published is that of Watanabe et al. (1975). They infected 20-day old chicks with reovirus orally. Twenty days later, they detected virus-neutralising activity in the gut, which they ascribed to IgA.

2.6.3. Cell-mediated immunity

The cell-mediated immune response of the chicken to infection with avian reovirus has not received as much attention as the humoral immune response. The limited studies have referred to the possible roles of T and B cells in reovirus infections or to immunosuppressive effects of avian reoviruses.

Kibenge et al. (1987) designed a study to show the effects of surgical and chemical immunosuppression on the reovirus-induced tenosynovitis. They observed that chicks infected with reovirus after they had been thymectomised and treated with cyclophosphamide had higher mortality and more virus was isolated from their tendons, when compared to those treated with cyclophosphamide alone, thymectomised or bursectomised. They concluded that recovery from reovirus infection probably involves both B- and T-cell systems but with the B-cell being more active in protection.

Using the wattle test to examine the delayed hypersensitivity (DTH) response, Islam and Jones (1989) were able to demonstrate a marked reaction in chickens tested 6 weeks pi. This clearly indicated that there is a cell-mediated immune response in chicken after reovirus infection.

Using monoclonal antibodies directed against B lymphocytes, T lymphocytes, and chicken Ia antigen, Pertile et al. (1996) obtained a more detailed picture of the cellular infiltrates during the various stages of viral arthritis. They observed that T

lymphocytes and plasma cells were the predominant inflamatory cell types found in the synovium following inoculation with reovirus. In the acute phase, T-cells, mostly CD8, were present in low numbers. Most activity was in the subacute phase with increased numbers of CD4 and CD8 T-cells. Also, there were aggregates of T-cells, IgM-positive B-cells, and plama cells. The chronic phase was characterised by large numbers of T-cells, primarily CD4, with few IgM-positive B-cells, and some lymphocytes staining positive for Ia. They concluded that the types, numbers, and activation level of lymphocytes present in the tarsal joints are similar but not identical to those seen in rheumatoid arthritis in man.

2.6.4. Immunosuppression

The first speculations about reovirus being immunosuppressive started with the frequent isolation of reovirus together with other infectious agents such as *Staphyloccocus aureus* (Kibenge et al. 1982; Kibenge and Wilcox, 1983).

Although the exact mechanism by which reovirus may cause immunosuppresion is not clear, its likely to affect the B-cell, T-cell, monocytes or a combination of any of them. Various reports have described field observations or experimental studies related to reovirus as an immunosuppressive agent but none has conclusively proved so.

van der Heide et al. (1983) described 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 avian reovirus and suggested that this indicated some degree of immunosuppression by avian reovirus. This was supported by Rinehart and Rosenberger (1983), who observed a four-times higher condemnation rate due to Mareks disease among chickens vaccinated simultaneously with HVT and reovirus than in those vaccinated with HVT alone. After this observation Rinehart and Rosenberger (1983) did an experiment to verify the

proposal that the T-cell system is affected by reovirus infection. They looked at the effects of five strains of avian reoviruses, including a non-pathogenic, moderate and a highly virulent strain, on the immune functions of chickens. They found that avian reovirus 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 was either decreased or elevated depending on the strains of reovirus used.

In contrast, Cho (1979) observed that pre-exposure of chickens to avian reovirus reduced the incidence of gross lesions and or mortality due to experimental Marek's disease. This was followed by Cook and Springer, (1983) who reported that avian reovirus did not adversely affect the immunocompetance of 8 to 12 weeks old chickens as evaluated by haemagglutinating response to sheep red blood cells and delayed hypersensitivity response to PHA. Eidson and Kleven (1983) also aggreed that reovirus did not affect the protective ability of HVT against Marek's when administered simultaneously with reovirus.

Although they found reduced responses of peripheral blood leucocytes to PHA and reduced phagocytosis by monocytes, Montgomery et al. (1986) suggested that serologically there was no significant support for the hypothesis that avian reovirus could cause general immunosuppression.

A commonly agreed on factor is that reovirus may cause temporary CMI unresponsiveness (Hill et al. 1989; Montgomery et al. 1986; Pertile et al., 1995) but whether this causes immune suppression is yet to be established. Avian reoviruses have been shown to grow in monocytes *in vitro* by Bulow and Klaven, (1983), Mills and Wilcox, (1993) and this is could be one way virus may temporarily intefere with the immune response.

In an attempt to explain the mechanisms by which avian reovirus infection may cause depressed *in vitro* proliferative responses to spleen cells by T cell mitogens, Pertile et al. (1995), showed that

macrophages in the spleen of infected chickens were present in a "primed" state and produced increased levels of nitric oxide. The presence of these primed macrophages correlated with depressed in vitro T-cell mitogenesis. In their recent study, Pertile et al. (1996) showed that reovirus infection in chickens does not compromise the functional capabilities of T-cells but induces suppressor macrophages that inhibit T-cell functions.

2.6.5. Passive immunity

Chicks hatched from eggs laid by hens immune to reovirus, receive maternal antibodies from the yolk and this has been the basis for protection of young chickens by breeder vaccination (Cessi and Lombardini, 1975; van der Heide, 1975; van der Heide et al., 1976; Eidson et al., 1979; Rau et al., 1980; van der Heide and Page, 1980; Thornton and Wood, 1981; Wood and Thornton, 1981, 1982; van der Heide et al., 1983).

2.6.6. Inteferon

Interferons are a family of proteins secreted by body cells in response to viral infection and are rapidly produced from within hours of infection and continue to be produced throughout the infection (Joklik, 1983). Bulow et al. (1984) reported avian reovirus-induced activation of cultured chicken bone marrow-derived and blood monocyte-derived macrophages and they suggested that the macrophage activation was mediated by endogenous interferons.

2.6.7. Auto-immune response

Avian reovirus-induced arthritis has been suggested to be an autoimmune disease which could be a useful model for human rheumatoid arthritis (Walker et al., 1977; Marquardt et al., 1983), but attempts to demonstrate the rheumatoid factor have been unsuccessful (Taylor, 1965; Walker et al., 1977). Other indications that reovirus-arthritis in chickens might be due to autoimmune reactions were reported by Pradhan et al., (1987) and Islam et al.

(1990) who were able to demonstrate the presence of antinuclear antibody in the serum of reovirus-infected chickens.

2.7. Diagnosis

2.7.1. Clinical signs

The clinical signs usually seen in cases of tenosynovitis are not pathognomonic. Cases of viral infections involving the respiratory tract, viral diarrhoea, unthriftness and hepatitis could be considered in the differential diagnosis. The disease should however be suspected in cases of lameness especially with involvement of the hock joints. Tenosynovitis has been described as primarily affecting meat-type chickens, although light breeds have been affected as well (Schwartz et al., 1976). Tests for reovirus detection are available but due to the widespread nature of reovirus infection, results are often difficult to interpret.

2.7.2. Virus demonstration

Suspected cases of avian reovirus infection should be confirmed by isolation of virus. The most satisfactory method is to use chick embryo liver cells (CELi) (Guneratne et al. 1982) (see Chapter 3). Tissues are macerated in medium M199, Eagle's MEM or nutrient broth with 10% concentrated antibiotics. After centrifugation the supernantants are used to inoculate confluent tissue culture monolayers. Avian reoviruses will replicate in the CELi cytoplasm and produce characteristic syncytial-type CPE. Other tissue culture cells in use are CELu and CK. Isolation can also be perfomed in embryonated chicken eggs of 5-6 days incubation by inoculation of material into the YS, 9-11 days old by the AC or the CAM. A characteristic feature of reovirus-infected embryos is yellowish-green necrosis of the liver (Guneratne et al., 1982). For all these cultures, field samples may require more than one passage before virus replication is detected.

Further characterisation of virus may be done by electron microscopy and serum neutralisation. Immunofluorescence staining of affected joint samples may also be used for a rapid diagnosis (Olson, 1975) but it may only be effective with fresh tissues and early in the infection.

2.7.3. Serological diagnosis

Serological tests have been used widely to detect positive reactors to infection or to evaluate the immune response after immunisation. Unfortunately, none of the tests differentiates between infections with virulent and non-virulent virus strains.

The earliest described and simplest test for antibodies to reovirus is the AGP test which detects group antigens for avian reovirus. The test uses high-titre virus usually grown on the CAM or in cell-culture (Olson,1975).

Virus neutralization tests in cell culture have been used and are more precise in detection of antibodies to particular serological types (Kawamura et al., 1965; Sahu and Olson, 1975; Sahu et al., 1979; Wood et al., 1980; Robertson and Wilcox, 1984). Ide and Dewitt (1979) suggested a combination of the AGP and neutralisation test for field assesment of reovirus infection.

An indirect IF test using infected cell cultures has been described by Ide (1982) performed in plastic micro-culture plates. The indirect IF test was compared with AGP in the detection of antibodies to avian reovirus in chickens, turkeys and duck sera (Adair et al., 1987). It was found to be more sensitive for group-specific antigen detection.

Further advances in the detection of avian reoviruses by indirect IF have come about as a result of the production of monoclonal antibodies (Mills and Wilcox, 1993; Shapouri et al., 1996).

Similarly the use of monoclonal antibodies has been extended to immunoperoxidase staining by Laniqing et al. (1996).

A western-blot method was also compared to the IF test and was found to be more sensitive (Endo-Munoz,1989). It is however a complex test to perform and is unlikely to find use as a routine test.

ELISA techniques have been developed for the diagnosis of infections of detection of the immune response after vaccination with avian reovirus (Slaght et al., 1978: Islam and Jones, 1988; Giambrone and Solano, 1988) and commercial kits are now available. For testing of large number of sera, ELISA is the test of choice, but it is group-specific.

2.8. Prevention and control

The fact that reovirus infections of chickens are widespread in nature, that there is no treatment, that the virus is fairly resistant to commonly used disinfectants and that infection can be transmitted vertically, leaves the use of vaccines as the main control measure for reovirus-induced tenosynovitis.

Because chickens are most susceptible to reovirus infection immediately in the post-hatching period and become resistant with age (Jones and Georgiou, 1984; Kataria et al., 1988; Roessler and Rosenberger, 1989), efforts to control tenosynovitis in chickens have concentrated on protection of the chicks early in life. Development of vaccines has therefore been aimed at conferring protection to the chicks either in the form of passive immunity by maternal antibody following vaccination of the parents, or by active immunity after early vaccination.

Initial attempts to prevent early infection and control tenosynovitis by vaccination were based on controlled exposure of 1-day old chickens to live virus (Mandelli et al., 1969; van der

Heide, 1973). These attempts provided variable results, apparently due to the pathogenicity and lack of cross-protection of some strains. van der Heide (1973) and Olson (1975) described the vaccination of 1-day old chicks with an attenuated strain S1133, which itself induced some tenosynovitis, although a degree of protection against challenge with strain UMI-203 was evident. Eleazor (1980) reported the use of a non-pathogenic vaccine which was given subcutaneously to 1-day old chickens, with or without maternal antibody and provided protection up to 10-17 weeks of age, by which time they could be vaccinated with a broiler-breeder vaccine.

Vaccination of one-day old broilers with strain WVU-1675 resulted in a significant reduction in the incidence of tendon involvement (Olson and Sahu, 1975). However, the vaccinations of 1-day old chickens with Reo-25 produced a 30% mortality and that with S1133 produced some tenosynovitis (van der Heide, 1973). Some protection to challenge by foot-pad with the Maine isolate UMI-203 was observed in the S1133 inoculated chickens.

In order to avoid problems associated with active vaccination of day old chickens, efforts were later directed towards administration of live and or inactivated vaccines to breeding flocks, to provide passive immunity to progeny via the yolk sac (Higgins, 1975; van der Heide et al., 1976). Cessi and Lombardini, (1975) reported a vaccination procedure using an inactivated vaccine prepared from strain S1133. A similar inactivated S1133 vaccine was used in commercial broiler breeders and SPF white Leghorn breeders (Wood and Thorton, 1981; Thorton and Wood, 1982).

Short-lived persistence of immunity was noticed as a serious drawback with this type of vaccine because the maternal antibodies decreased steadily after hatching, and by 3 or 4 weeks of age they were no longer detectable (van der Heide, 1977; Rau et al., 1980; Naqi et al., 1983). van der Heide et al (1975) described the vaccination of broiler breeders by intramuscular inoculation of a live non-attenuated strain of S1133. One day old chickens from these breeders were resistant to oral but not

subcutaneous challenge. Later, an attenuated version of S1133 strain was used and the progeny also showed a resistance to oral but not to subcutaneous challenge with a virulent form of the homologous virus (van der Heide, 1976).

Eidson et al. (1979) conducted similar trials using the 74th embryo passage of the S1133 strain and claimed that the progeny were more resistant to both oral and subcutaneous challenges than those from unvaccinated flocks. van der Heide and Page (1980) used this same 74th embryo passage of strain S1133, administered by drinking water to broiler breeder flocks and monitored precipitating antibody development in these parents. AGP antibodies were detected 3 weeks after vaccination in two flocks, but the third flock did not develop any antibodies until 15 weeks after vaccination, presumably due to natural exposure. The failure of the vaccine to take was attributed to high chlorine levels in the drinking water used by this flock at the time of vaccination. Progeny from the two successfully vaccinated breeders flocks remained free from tenosynovitis while the third breeder flock developed tenosynovitis. A similar vaccine (73th embryo passage of S1133) was also shown to be effective by eye-drop vaccination but was less effective when applied at a higher passage (175th) and via drinking water (Rau et al., 1980).

A wide cross-protection against antigenically different viruses is an ideal property of a good vaccine. To determine the degree of cross-protection against heterologous viruses in chicks from S1133 vaccinated breeders, Rau et al. (1980) orally challenged one-day old chicks from vaccinated breeders with the homologous virus and five heterologous avian reoviruses. They found that chicks were resistant to challenge by the S1133 virus and four of the heterologous viruses. The chicks were not resistant to strain Reo-25, an indication of lack of protection against this strain by S1133.

There are reports of insufficient protection by S1133 reovirus strain to challenge with avian reovirus isolates WVU-2937, UMI-203 and Texas (Springer et al., 1982).

In yet another development, Jones and Nwajei (1984) reported the use of a 73rd embryo passage of S1133 in broiler hens and found that 50% of their progeny were protected after oral challenge at 1-day old with a virulent form of the same virus. It was, however, found that the protection provided by maternal antibody extended to the joints, but did not stop challenge virus being excreted via the intestinal tract. Presumably the protection was due to circulating antibody and not antibody in the gut.

In an attempt to reduce some of the undesirable effects of live vaccines, van der Heide et al. (1983a) developed a fully-attenuated reovirus vaccine based on the S1133 strain by 235 serial passages in chick embryos followed by 100 additional passages in CEF cultures. This attenuated vaccine was given by the subcutaneous route to one-day old chickens with or without maternal antibodies, which were then challenged via the foot-pad with homologous virus 14 days later. Vaccinated groups with or without maternal antibodies were found resistant while unvaccinated control groups were not.

Another attenuated live reovirus vaccine, prepared from the UMI-203 isolate by passage 90 times in tissues culture and used in breeders of 8 to 18 weeks of age was used in the USA (Haffer, 1984). This vaccine however was highly pathogenic for the chickens used in their work which were between 8 and 18 weeks old. From this 90th passaged isolate, Haffer, (1984) developed a temperature-sensitive mutant which he further passaged 19 times. The UMI-203 mutant was found to be a good candidate vaccine for prevention of early tenosynovitis infection in chickens because of its safety, lack of suppression of other vaccinations, and cross-protection potential.

Four modified live reovirus vaccines were compared for safety by use of a field isolate (81-176) reovirus for challenge in 1-day old and 1-week old SPF chicks (Montgomery and Maslin, 1988). The vaccine efficacy was monitored by challenging chickens seven weeks post infection via the foot pad. For the chickens vaccinated at one day of age, three of the four vaccine were consistently

recovered from the hock tendon tissues. Post-challenge virus recoveries were similarly positive for the same vaccine viruses. In contrast, in the chicks vaccinated at one week of age, none of the vaccine viruses was recovered from any of the vaccinated groups.

The use of a coarse-spray (CS) administration of a commercial S1133 reovirus vaccine (Enterovax) was evaluated in SPF chickens and commercial broiler chickens. The aims were (i) to establish the efficacy of CS application, (ii) to determine whether it is likely to interfere with the efficacy of other vaccines, and (iii) to compare CS with D W (Giambrone and Hathock, 1991). It was found that both drinking water and CS administration were efficacious and there was no interference with other vaccines. However, virus-neutralising antibodies to reovirus could not be detected in 1-day old vaccinated chicks but could only be detected after a booster vaccination at seven days of age by CS or DW.

Giambrone et al. (1991) found that after giving a live vaccine based on a cell-cultured clone of strain S1133/66 (Enterovax) to adult broiler breeder hens via the drinking water, there was an increase in the antibody response compared to an inactivated vaccine.

Management procedures designed to decrease the extent of reovirus exposure of young birds may be of some use in the control of tenosynovitis. These include all-in-out site policies thorough cleaning and disinfection of houses between successive crops of broilers and good hygienic practices (McFerran and McNulty, 1993).

CHAPTER 3

GENERAL MATERIALS AND METHODS

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CHAPTER 3

GENERAL MATERIALS AND METHODS

This section describes the general materials and methods used routinely while undertaking this work. Specific techniques used in certain experiments appear in the relevant sections in which they are applied. Methods for preparing all reagents, buffers and media used in these studies will be given in the Appendix.

3.1 Glassware

All items of glassware were collected and cleaned according to the following procedure in the laboratory before they were used.

3.1.1 Contaminated glassware

All glassware that was used with any infectious material was soaked overnight in a disinfectant before cleaning and sterilization. Both graduated and pasteur pipettes were soaked in sodium hypochlorite (Chloros, Golden Grain Products Ltd., Liverpool, England). Pasteur pipettes were discarded and graduated pipettes were immersed in a liquid detergent (Micro, International Products Corp., Chiselhurst, Kent, England) overnight after their cotton wool plugs were removed. They were then rinsed in tap-water, then distilled and deionized water. After use, all pasteur pipettes were discarded into safety buckets.

The rest of the glassware which had been used with contaminated materials was collected in stainless steel buckets and autoclaved at 121^0C for 30 minutes at 15 lb./sq.in. All used disposable plasticware was put in plastic bags after disinfection by overnight

soaking in an iodophor-based disinfectant (FAM, Evans Vanodine, Manchester, England). The plastic bags were sealed and incinerated.

3.1.2 Cleaning of glassware

All glassware which was new or had been disinfected was cleaned either manually and rinsed, or automatically using a glass washing machine (Gallay Lab 901, Jean Gally and Co. Ltd., Hemel Hempstead, Herts, England). Manual cleaning was by brushing or scrubbing. Glassware was soaked in a liquid detergent (Micro) overnight, then cleaned thoroughly and rinsed serially in changes of tap, distilled and distilled-deionized (DD) water. The clean glassware was inverted in wire baskets for the water to drain out and dried in a hot air oven. The glassware was washed in the washing machine, with a detergent (Gallay clear"N"), rinsed in tap-water then distilled and deionized water and dried automatically.

3.1.3 Sterilization of glassware

Bottles were closed with plastic or metallic caps. Flasks, measuring cylinders and test tubes were closed with aluminium foil. Graduated and pasteur pipettes were plugged with cotton wool and sterilized in stainless steel containers in a hot air oven at 160^0C for two hours. Pestles and mortars and griffiths tubes were wrapped in plastic bags and autoclaved at 121^0C for 30 minutes at a pressure of 15 lb./sq. in. All autoclavable glassware was dried thoroughly in a drying oven.

3.2 Source of chickens and fertile eggs

The specific pathogen free (SPF) eggs were obtained from a commercial supplier (Wickham laboratories, Wickham, Hants, UK). The parent flock of white leghorns was free of the major infectious disease agents including reovirus and chicken anaemia virus.

All eggs were washed with eggshell disinfectant (Ryclor, Ltd. Oakenshaw Chemical Works, Clayton-Le-Moors, Accrington, England) in an electrical egg washing machine (Rotomaid, Vantec Ltd., Market Drayton, Salop, England). Eggs were dried, stored at room temperature and used within one week of being laid. Eggs were incubated in automatic incubators, with 400 eggs capacity. The eggs to be used for production of hatched chicks were transferred from the setter section of the incubators to the hatching compartment on the 18th day of incubation.

3.3 Housing and management of chickens

All experimental chickens were housed in the department's isolation unit. The isolation unit is located about 50 yards away from the laboratory. The unit comprises ten individual pens, one large anteroom, and a corridor in between the pens. All rooms are supplied with negative pressure filtered air. Each pen is supplied with mains water. Footbaths containing disinfectant (2% FAM in water) are put at the main entrance and on the entrance of each of the pens.

During each experiment visiting the isolation unit for purposes of observation of birds, sampling or cleaning are restricted to only persons carrying out the necessary duties. The protocol to enter the isolation unit is by use of a one way-system, that is, one door for entry only and one for exit only. Before entering the unit protective clothing which includes overalls, head and face masks, gloves have to be worn. Before exit, all the protective clothing is removed and soaked in a drum of disinfectant.

All the material and equipment used within the pens are wrapped in plastic bags before entrance. Chicks to be taken in are also placed in ventilated boxes which are loosely protected by a plastic bag.

Experimental birds are kept in the isolation unit from day-old up to the end of the experiment. Food and water are given birds *ad*

libitum. Minor sampling procedures such as swabbing or bleeding which do not warrant killing of the birds are carried out within the pens. For major procedures such as tissue sampling, birds are humanely killed and placed in clean boxes, covered by plastic bags and taken to a post mortem room which is located outside the isolation unit.

3.4 Cell cultures

3.4.1 Cell culture and supplements

Throughout this study, primary chicken embryo liver (CELi) cell cultures were used for investigations involving virus isolation, titration and replication. The medium used for cell cultures was M199 (Appendix-A) supplemented with 0.01M sodium bicarbonate, 10%(v/v) tryptose phosphate broth (TPB), 0.014M HEPES buffer (n-2-hydroxyethyl piperazine-N-Z-ethane sulphonic acid) pH 7.0, antibiotic solution (100µg streptomycin and 100 i.u. penicillin per ml of medium). Newborn calf serum (Gibco Ltd., Paisley, Renfrewshire, Scotland) was added to a concentration of 10% for growth medium or 5% for maintenance medium.

3.4.2 Cell culture procedure

Chicken embryo liver cell cultures were prepared from 14-day old SPF chicken embryos as described by Jones and Kibenge (1984). For each egg, the area of the shell over the air space was disinfected with 70% alcohol. Using sterile instruments, a circular area over the air sac was cut out and the embryo was removed to a petri dish where it was killed by decapitation using a pair of scissors and a pair of forceps.

The livers were removed without the gall bladder, using curved-ended forceps and collected in phosphate buffered saline A (PBS-A) (Appendix-A). They were cut into small pieces and collected in pre-warmed PBS-A in a 100ml side-arm flask. Approximately 100ml of pre-warmed 0.05% trypsin solution in PBS-A (Appendix-A) was added to the liver pieces and incubated for five to ten

minutes at 37^{0}C with a magnetic stirrer, to facilitate the disaggregation of the tissues. After this cycle of trypsinization, the tissue in the flask was removed from the incubator, allowed to settle and the supernatant was poured slowly into four universal bottles, each containing 2ml of pre-cooled calf serum. This procedure of trypsinization was repeated three to five times and ice cubes were put around the universals in a plastic box to prevent further digestion.

The supernatants were centrifuged at 800 rpm for five minutes to sediment the cells from the suspension. The cell pools were resuspended in a 10 ml growth medium. The suspension was mixed gently and sedimentation was repeated by centrifugation at 800 rpm for five minutes. All cells were finally resuspended in 10ml of fresh growth medium. A viable count was done on the cells using a haemocytometer using 0.1% trypan blue to indicate viability. The final cell concentration was adjusted according to the seeding requirement.

3.4.3 Seeding rates

The seeding rates of CELi cells in tissue culture plasticware (Gibco Ltd., Paisley, Renfrewshire, Scotland) for virus were as follows:

Vessel	Capacity	Cells/ml
25cm flask	8ml	$1x10^6$
75cm flask	25ml	$1x10^6$
175cm flask	50ml	$1x10^6$
96-well plate	0.1ml/well	$1x10^6$
48-well plate	0.5ml/well	$0.5x10^6$
24-well plate	1ml/well	$0.5x10^6$

All vessels containing cells were incubated at 37^{0}C in a humid environment with 5% CO_2 in air. Confluent cell monolayers were usually formed within 48 hours.

3.5 Viruses and virological methods

3.5.1 Source of viruses

Two strains of avian reovirus, both UK isolates were used. One was reovirus R2 which is trypsin-resistant and the second, TR1 which is trypsin-sensitive.

Strain R2 was originally isolated from an outbreak of tenosynovitis (viral-arthritis) in broiler breeders (Jones et al., 1981) and has been shown to cause tenosynovitis with rupture of digital flexor tendons in SPF light breed chickens and gastrocnemius tendons in commercial broiler chickens (Jones and Kibenge, 1984). It has been passaged 17 times in CELi cell cultures without loss of pathogenicity. Strain TR1 was originally isolated from an outbreak of lameness in 12-week-old commercial turkeys and is pathogenic for chicks (Al-Afaleq and Jones, 1991). TR1 has been passaged 18 times in CEli cells without loss of pathogenicity.

The trypsin resistance of strain R2 and the trypsin sensitivity of TR1 have been documented (Al-Afaleq and Jones, 1991; Jones et al., 1994).

3.5.2 Preparation of tissue samples for virus isolation

Tissue samples were collected aseptically using sterile instruments and weighed. Separate tissues were kept in small plastic sample bottles and immediately processed. They were cut finely on a petri dish using sterile scissors or scapel blade and transferred to a sterile mortar where they were ground with a pestle. A small volume of sterile sand was added to facilitate the processing. A small amount of medium (M199) containing x10 the normal concentration of penicillin and streptomycin (Appendix-A) was added to clean the surface of the mortar from all remaining tissue. The tissue and medium were transferred to a universal bottle. The total volume of medium added to each sample was in proportion to the weight to give a 10% (w/v) suspension. The prepared

tissues were frozen and thawed three times, followed by centrifugation at 2500 rpm for 10 minutes to remove the large debris and the supernantant was collected and kept at 70^0C.

3.5.3 Preparation of cloacal swabs samples

After collection, each cloacal swab was added to 2ml of M199 medium with x10 penicillin and streptomycin in a 5ml plastic bottle which was agitated vigorously and kept in -70^0C until used. Swabbing of chicks in their first week of life was made less traumatic by first moistening the swabs in medium.

3.5.4 Infection of CELi cell cultures

When CELi monolayers in either 48 well plates or 75cm flasks were confluent, the growth medium was removed using a pasteur pipette connected to a vacuum pump (48 well plates), or poured off (75cm flasks). Two drops or 50µl of each sample supernatant were inoculated onto the CELi monolayers in each of two replicate wells of a 48 plate or in one 75cm flask. Virus adsorption was allowed for 1 hour at 37^0C after which 0.5 ml or 25ml of fresh maintenance medium was added to the respective well or flask. The cultures were incubated for up to 7 days and examined daily for the appearance of typical reovirus syncytial CPE.

Samples showing no CPE on first passage were subjected to three to four cycles of freezing and thawing followed by vigorous pipetting to disrupt the cells and release the virus. In the case of 48 well plates, the cell culture fluids of the replicate wells were mixed and two drops were reinoculated into fresh CELi cell culture monolayers as done before. For the 75cm flasks, 0.5ml of the disrupted cell culture fluid was used to reinoculate fresh 75cm flasks as done before.

All apparently negative samples were further passaged to a total of three times and if they still showed no CPE, then they were considered negative. From time to time, positive samples were checked for reovirus by negative staining with phosphotungstic acid and examined under a Philips 301 electron microscope.

3.5.5 Virus titration

Virus titration was performed in 96-well microtitration plates using CELi cell cultures. The titrations followed the method described by Grimes et al. (1976).

Serial ten-fold dilutions of virus were made using M199 stock medium without calf serum in bijou bottles. Fifty microlitres of growth medium were pipetted into all the wells in the plate followed by the application of 50µl of each virus dilution. Five horizontal rows were used for replicates of each dilution from 10^1 to 10^8 dilutions. Two columns of the plate were selected as cell controls and to them were added 100µl of growth medium. Finally, 50µl of fresh CELi cell suspension, containing $1x10^6$ cells per ml was added to all the wells in the plate, including the two uninfected controls columns. Plates were incubated at 37^0C with 5% carbon dioxide in a humidified incubator and examined daily for appearance of CPE using an inverted microscope. On the 7th day of incubation the result was recorded as the highest dilution showing CPE for each replicate. The end-point titre was expressed as the 50% tissue culture infective dose ($TCID_{50}$) calculated by the Reed and Muench (1938) method.

3.6 Enzyme-linked immunosorbent assay (ELISA) for immunoglobulin assay

3.6.1 Purification of virus. CELi cells in 75 cm tissue culture flasks were infected with 200µl of medium M199 containing approximately 4.0 log_{10} $TCID_{50}$ per 50µl of stock avian reovirus strain R2. After incubation for 3 to 4 days, when the maximum CPE was achieved, the flasks were shaken and frozen at -70^0C and then thawed at 37^0C. This was repeated at least three times in order to release virus. Cellular debris was removed by centrifugation at 2000g for 20 minutes in a bench centrifuge. The supernatant was centrifuged again at 50,000g for 90 minutes at 4^0C in an MSE high speed ultracentrifuge (MSE Scientific Instruments, West Sussex, England). After this, the supernatant

was poured off and the pelleted virus was resuspended overnight at 4^0C in a small amount of 0.05M Tris, 0.01M NaCl, 0.001M EDTA (TNA) buffer, pH 7.8 (Appendix-A). The virus was then resuspended gently and layered onto a two-layer discontinous caesium chloride gradient (Sigma, Ltd, Poole, Dorset, UK) consisting of 4.5 ml of 1.6 g/ml and 15 ml of 1.25 g/ml 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 4^0C in a swing-out rotor in the ultracentrifuge.

From the tube, 1 ml lots of the gradient were collected, taking care not to disturb the diffuse band at the junctions of the two density solutions. The densities of each of these fractions were determined and those between 1.3 and 1.5 were selected and resuspended overnight at 4^0C in 0.05M Tris-HCl buffer and repelleted by centrifugation at 50,000g for 90 minutes at 4^0C. The pellet was finally resuspended at 4^0C in 0.05M Tris-HCl buffer to give a 100-fold concentration of the original tissue culture fluid. This antigen was divided into small aliquots and the protein concentration measured using BCA protein assay reagents (Piece Chemical Co., Rockford, Illinois, USA). The antigen was used at a concentration of 5µg per ml.

3.6.2 Buffers. Phosphate-buffered saline (PBS)-Tween was used as washing buffer. Freshly made 0.1 M carbonate/bicarbonate (CBC), pH 9.6 was the coating buffer. PBS-Tween with 5% powdered skim milk was the diluent, while the substrate solution was freshly prepared 0.01% o-phenylenediamine (OPD) (Sigma) activated with 0.015% hydrogen peroxide in 0.01M phosphate buffer at pH 7.0.

3.6.3 Monoclonal antibodies (Mabs). These were tissue culture fluids specific for chicken IgA (p9) and a pool of IgG (G1+G3+G9), obtained from Dr T. F. Davison, Institute for Animal Health, Compton, UK.

3.6.4 Conjugate. Horseradish peroxidase-conjugated goat anti-mouse IgG (whole molecule) was obtained commercially (Sigma).

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3.6.5 ELISA procedure. An indirect ELISA as described by Myers and Schat, (1989) was used with some modifications. Briefly 50µl of purified antigen were placed in alternate wells of a 96-well ELISA plate (Falcon, Becton Dickinson Labware, Oxnard, California, USA). CBC was placed in the other wells and the plate incubated at 4^0C overnight. Plates were washed three times between reactions and other reagents were used as 50µl volumes and incubations done at 37^0C for 1 hour. After the initial incubation, test samples were pipetted onto the plate followed by the anti-immunoglobulin Mab and conjugate. Following checkerboard titrations, intestinal contents were used diluted 1:10, and sera 1:50. The Mabs were applied at a dilution of 1:10 while the conjugate was used at 1:400.

Phosphate buffered saline (PBS)-Tween was used as washing buffer. Freshly made 0.1M CBC, pH 9.6 was the coating buffer. PBS-Tween with 5% powdered skim milk was the diluent, while the substrate solution was freshly prepared 0.01% o-phenylenediamine (OPD) (Sigma) activated with 0.015% hydrogen peroxide in 0.01m phosphate buffer at pH 7.0.

The reaction was stopped when the absorbance in the darkest wells of the control sample reached an optical density value of approximately 0.1 by adding 25µl of 2.5N sulphuric acid. The plate was read by an automatic ELISA reader (model MR700, Dynatech, Billingshurst, Sussex, UK) which was set up to read at 490nm (test filter 3) with a reference reading at 630 nm (reference filter 5).

CHAPTER 4

INTESTINAL AND SYSTEMIC ANTIBODY RESPONSES TO AVIAN REOVIRUS: THE INFLUENCE OF AGE AT INFECTION, ROUTE OF INOCULATION AND TRYPSIN-SENSITIVITY OF THE VIRUS

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CHAPTER 4

INTESTINAL AND SYSTEMIC ANTIBODY RESPONSES TO AVIAN REOVIRUS: THE INFLUENCE OF AGE AT INFECTION, ROUTE OF INOCULATION AND TRYPSIN-SENSITIVITY OF THE VIRUS

4.1 Introduction

Avian reoviruses have a world-wide occurrence and are ubiquitous in commercial poultry. Tenosynovitis in chickens is the most important disease attributed to avian reovirus (Kerr and Olson, 1967; Glass et al., 1973; Jones et al., 1975; Jones and Georgiou, 1984) and the disease occurs predominantly in heavy breeds although it has been reported in commercial white leghorn pullets (Schwartz et al., 1976; van der Heide, 1977). Although they have been isolated from clinically healthy chickens (Mustaffa-Babjee and Spradbrow, 1971; Mustaffa-Babjee et al., 1973), avian reoviruses have also been implicated as a cause of immunossuppresion in chickens (Springer et al., 1983; Montgomery et al., 1985). Thus both tenosynovitis and perhaps the subsquent infections due to immunosuppression may lead to losses as a results of poor growth and condemnations at slaughter. Although avian reoviruses can be egg-transmitted (Menendez et al., 1975; Al-Mufarrej et al., 1996) the main challenge to chicks is via the faecal-oral route after which initial replication of virus occurs, principally in the mucosa of the digestive and respiratory tract (Menendez et al., 1975; Ellis et al., 1983; Kibenge et al., 1985; Jones et al., 1989; Ni and Kemp, 1995). The intestinal tract is also a principle route of excretion of the virus (Jones et al., 1989).

Chickens are susceptible to avian reovirus infection from day-old and they develop resistance to infection with age (Jones and Georgiou, 1984). Protection of chicks is therefore essential either by maternal immunity or early vaccination.

Most current vaccines are derivatives of strain S1133 which has been shown to be sensitive to digestive enzymes (Jones et al., 1996). Such sensitive viruses replicate poorly in the intestines of chicks, even after oral infection (Jones et al., 1994). Live vaccines are given to young stock either parentally or orally and more recently the coarse-spray method has been used (Giambrone et al., 1992).

The stimulation of the secretory IgA system and the local production of IgA antibody is recognized as one of the first lines of defence against mucosal infections in the chicken (Schat, 1991).

The involvement of the humoral arm of the mucosal immune system following exposure to various intestinal mucosal diseases in chickens has been reported to induce protection in certain poultry diseases, including coccidiosis (Davis et al., 1978), Newcastle disease (Kono et al., 1969; Lee and Hanson, 1975; Jayawardane and Spradbrow, 1995), *Escherichia coli* infection (Parry et al., 1977), salmonellosis (Hassan et al., 1991) and rotavirus infection (Myers and Schat, 1990). Although avian reoviruses are known to replicate in the intestinal tract (Menendez et al., 1975; Ellis et al., 1983; Kibenge et al., 1985; Jones et al., 1989; Ni and Kemp, 1995), the local immunity to reovirus infection in the gut has not been explored.

It is thought that an understanding of the role of the intestinal local immunity to reovirus may contribute to formulation of vaccines which will induce better gut immunity.

The main objectives of this study were as follows. Part I: To investigate the local and systemic immune responses in chicks after infection with the trypsin-resistant strain R2 in birds of three ages (Experiments 1, 2 and 3). Part II: Using the trypsin-sensitive strain TR1 as a corollary to strain S1133, to study the local and systemic immune responses after oral or subcutaneous infection in two different age groups (Experiments 1, and 2).

PART I

INFECTION WITH A TRYPSIN-RESISTANT VIRUS

EXPERIMENT 1. COMPARISON OF ORAL AND SUBCUTANEOUS INFECTION OF DAY-OLD CHICKS.

4.2 Materials and methods

4.2.1 Chicks

One hundred and five one day old hybrid SPF chicks were used. They were given water and food *ad libitum.*

4.2.2 Viruses

The trypsin-resistant avian reovirus strain R2 and the trypsin-sensitive strain TR1, already described by Al-Afaleq and Jones (1991) were used (see Chapter 3).

4.2.3 Experimental design

Chicks were divided into three groups of 35 chicks each and kept in separated isolation rooms. One group was infected orally by oesophageal cannulation with 0.3ml of M199 medium containing 3.5 $\log_{10}$ $TCID_{50}$ per 50µl of reovirus R2. A second group was infected with a similar quantity of the virus but subcutaneously in the loose skin of the back of the neck. The third group was sham-infected orally with M199 only and kept as controls.

Five chicks from each experimental group were first bled and thereafter killed by CO_2 asphyxiation on days 1, 3, 5, 7, 10, 14 and 21 post-inoculation.

The intestinal tract was aseptically removed and various tissues were taken for virus isolation and antibody estimation (see below).

4.2.4 Intestinal tract sampling

The alimentary tract was removed between the gizzard-duodenal junction and the cloaca. Using separate sterile instruments, approximately 2.5 cm lengths of each of the duodenum, jejunum, ileum, caecum and rectum were collected and the like pieces of each region from each bird were pooled. From each piece of the gut, the intestinal contents were gently squeezed out, pooled and put into a pre-weighed plastic container and kept at -70^{0}C until immunoglobulin assays were done. The remaining piece of the gut was cut into smaller sections which were separately kept for virus isolation and titration.

4.2.5 Intestinal contents treatment

Intestinal contents were immediately reconstituted into a 10% PBS solution containing 50mM EDTA, 0.1mg m^{-1} soybean trypsin inhibitor (Sigma Chemical Company, Poole, Dorset, UK) and phenylmethylsulfonylfluoride (PMSF) (Sigma) in absolute ethanol (final concentration was 0.35mg ml^{-1}). After vigorous mixing on a whirlmixer and centrifugation, the supernatant was stored at -70^{0}C prior to testing by ELISA.

4.2.6 Virus isolation and titration

Samples from various pieces of the intestine were prepared as described in Chapter 3. The supernatants were inoculated on to CELi cell culture monolayers on 48 well plates. All negative samples were passaged up to three times in CELi cells.

Virus titration was performed on each of the supernatants of the gut region following the method described in Chapter 3. The final virus titre was calculated as $\log_{10}$ $TCID_{50}$ per 50µl of medium according to the method of Reed and Muench (1938).

4.2.7 ELISA for immunoglobulin assay

This was performed as already described in Chapter 3.

4.2.8 Virus-neutralisation test

This test was used on intestinal contents. The constant virus, varying serum (beta) method was used. In each well of a 96-well plate, except for the first column, 50µl of tissue culture medium were dispensed. One hundred microlitres of serum were pippetted into two wells of the first column. Using an automatic microdiluter, serial two-fold dilutions of the intestinal contents were performed in the subsquent wells up to the 10th column. Then, 50µl of virus suspension containing 100 $TCID_{50}$ were added to each well except those in the 12th column. The plate was agitated for five minutes on the plate shaker and incubated at 37^0C for two hours. After incubation 50µl of fresh cell suspension ($1x10^6$ cells per ml) were added to each well of the plate, which was then reincubated at 37^0C in a humidified atmosphere containing 5% CO_2. The 11th and 12th column served as virus and cell controls respectively. The plate was examined daily for evidence of CPE and results recorded, but the final reading was on day seven post-incubation. The neutralisation titre was considered to be the highest dilution of the test sample producing 50% or greater inhibition of visible viral CPE and expressed as the $\log_2$ of the reciprocal.

4.3 Results

4.3.1 Virus isolation and titration

Virus isolation

Tables 1 and 2 show the virus isolation results of the intestinal tissues from chicks infected orally or subcutaneously when day old with a trypsin-resistant avian reovirus strain R2. Isolation indices show the relative total virus isolations from each of the intestinal tissues.

Among the orally infected chicks there was no marked difference between the tissues except the caecum which showed the highest isolation index (Table 1), and almost all samples were positive throughout.

In the chicks infected orally, virus was first isolated on day 1 pi and apart from the caecum and duodenum no virus was isolated by day 21 pi. (Figure 1). Although virus was isolated by day 1 in the chicks infected by the subcutaneous route (Table 2), it was of a much shorter duration than in the orally infected chicks and by day 14 pi no virus was isolated except from the caecum and rectum.

Virus was isolated for a longer duration from the duodenum and caecum of chicks infected orally.

Table 1. Virus isolation results of pooled (n=5) intestinal regions of chicks infected orally when day-old with reovirus strain R2. Numbers 0, 1, 2, and 3 represent virus isolation indices following no isolation or isolation at the third, second or first passages respectively.

	Days pi							Isolation Index
Tissue	1	3	5	7	10	14	21	
Duodenum	3	3	3	3	2	0	1	15
Jejunum	3	3	3	3	0	0	0	12
Ileum	3	3	3	2	0	3	0	14
Caecum	2	3	3	3	3	3	3	20
Rectum	3	3	3	3	2	3	0	17

Table 2. Virus isolation results of pooled (n=5) intestinal regions of chicks infected subcutaneously when day-old with reovirus strain R2.

	Days pi							Isolation Index
Tissue	1	3	5	7	10	14	21	
Duodenum	0	2	3	0	2	0	0	7
Jejunum	3	3	3	1	0	0	0	10
Ileum	2	2	3	2	2	0	0	11
Caecum	1	2	3	2	0	1	3	12
Rectum	3	3	3	3	3	1	0	16

Virus titration

Virus titres in the intestinal tissues of chicks infected orally or subcutaneously when day old are shown in Tables 3 and 4, and Figures 1 to 5.

Both the oral and subcutaneous routes of infection had similar patterns of virus titres in the intestinal tract. However chicks infected orally had higher virus titres than those infected subcutaneously in the duodenum, jejunum, caecum and rectum.

Duodenum

After oral infection titratable virus was present between days 1 to 7 pi. After subcutaneous infection, virus was titratable only on days 3 and 5 and the peak on day 5 was less than 2.0 $\log_{10}$ lower than after oral infection (Figure 1).

Jejunum

In the jejunum similar patterns were seen after oral and subcutaneous infection, but titratable virus was present on day 1 after oral infection and peak titres were about 1.0 $\log_{10}$ higher (Figure 2).

Ileum

In the ileum, although titratable virus was present after oral infection sooner than after subcutaneous infection, higher titres were obtained in the latter (Figure 3).

Caecum

Virus titres in the caecum persisted twice as long after oral infection than after subcutaneous infection (Figure 4).

Rectum

Virus titres in the rectum were similar after both routes of infection but those following the oral route were higher on days 1, 3 and 7 pi (Figure 5).

Table 3 Virus titres (log_{10} $TCID_{50}$ per 50µl) in pooled intestinal tissues of chicks infected orally at day-old.

	Days pi						
Tissue	1	3	5	7	10	14	21
Duodenum	1.5	3.17	5.17	1.63	+	-	+
Jejunum	2.5	4.63	3.32	+	+	-	-
Ileum	3.17	2.5	2.5	+	+	+	-
Caecum	1.63	4.5	4.5	2.5	1.5	1.38	+
Rectum	2.5	4.63	4.0	3.5	+	+	-

+: positive, but titre <0.5 log_{10}; -: no virus isolated

Table 4 Virus titres (log_{10} TCID50 per 50µl) in pooled intestinal tissues of chicks infected subcutaneously at day-old.

	Days pi						
Tissue	1	3	5	7	10	14	21
Duodenum	-	2.69	2.83	-	+	-	-
Jejunum	+	3.63	2.63	+	-	-	-
Ileum	+	3.83	3.5	+	+	-	-
Caecum	+	4.0	3.17	1.32	-	+	+
Rectum	1.32	4.0	4.0	1.83	+	+	-

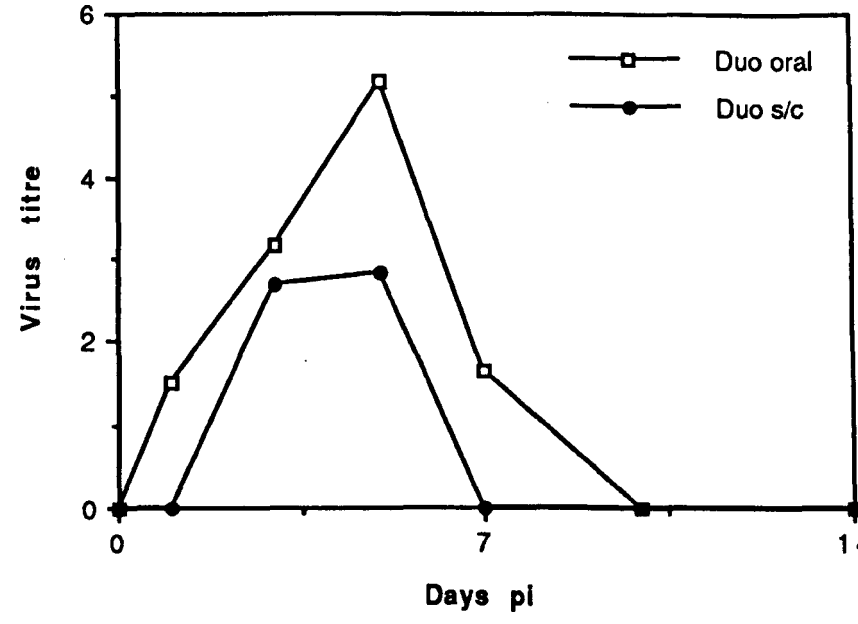

Figure 1. Virus titres (log_{10} $TCID_{50}$ per 50µl) in pooled duodenum of chicks infected orally or subcutaneously at day-old with a trypsin-resistant strain R2.

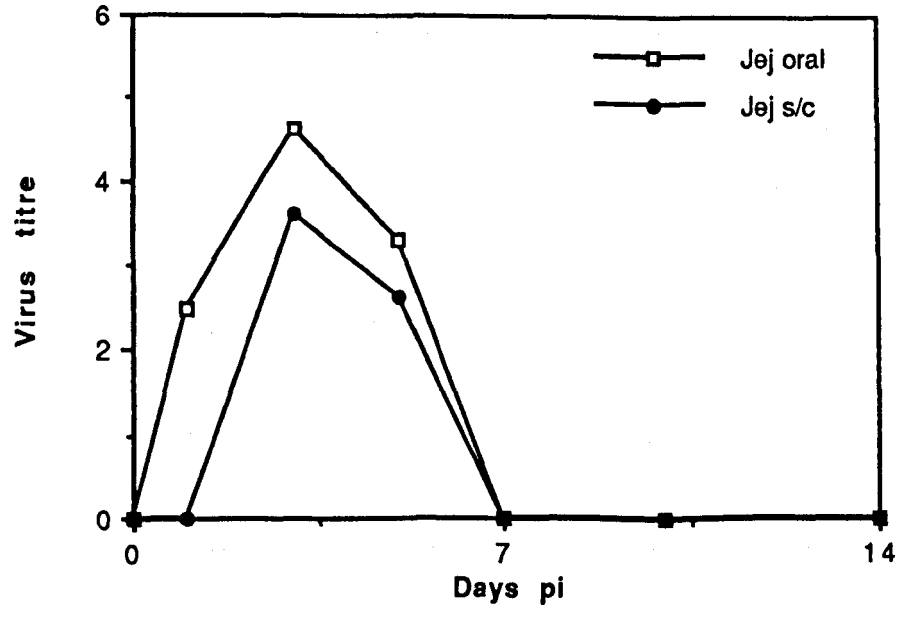

Figure 2. Virus titres (log_{10} $TCID_{50}$ per 50µl) in pooled jejunum of chicks infected orally or subcutaneously at day-old with a trypsin-resistant strain R2.

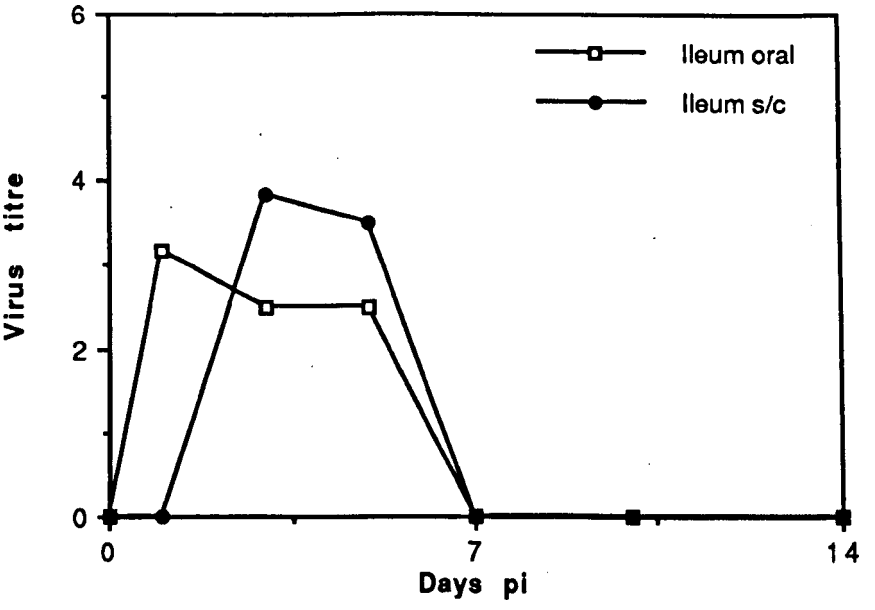

Figure 3. Virus titres (log_{10} $TCID_{50}$ per 50µl) in pooled ileum of chicks infected orally or subcutaneously at day-old with a trypsin-resistant strain R2.

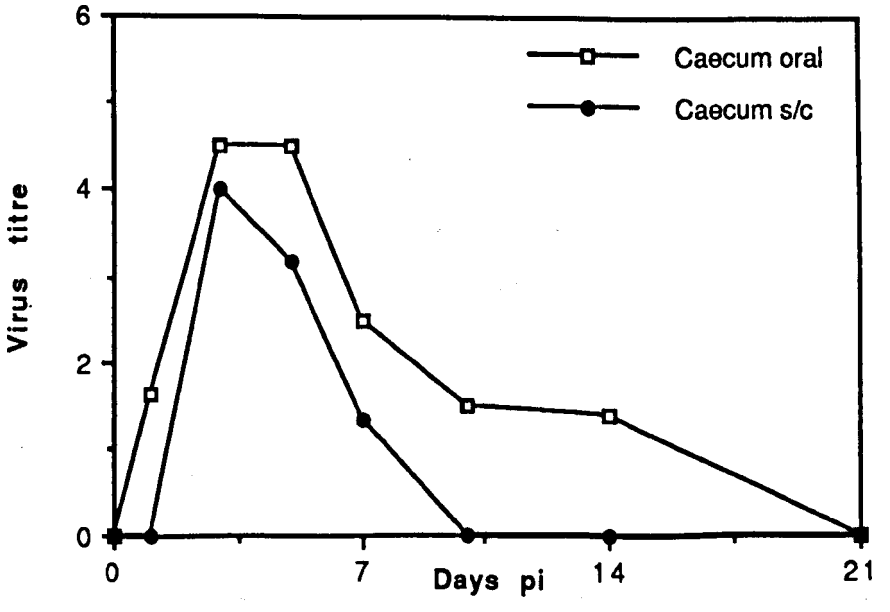

Figure 4. Virus titres (log_{10} $TCID_{50}$ per 50µl) in pooled caecum of chicks infected orally or subcutaneously at day-old with a trypsin-resistant strain R2.

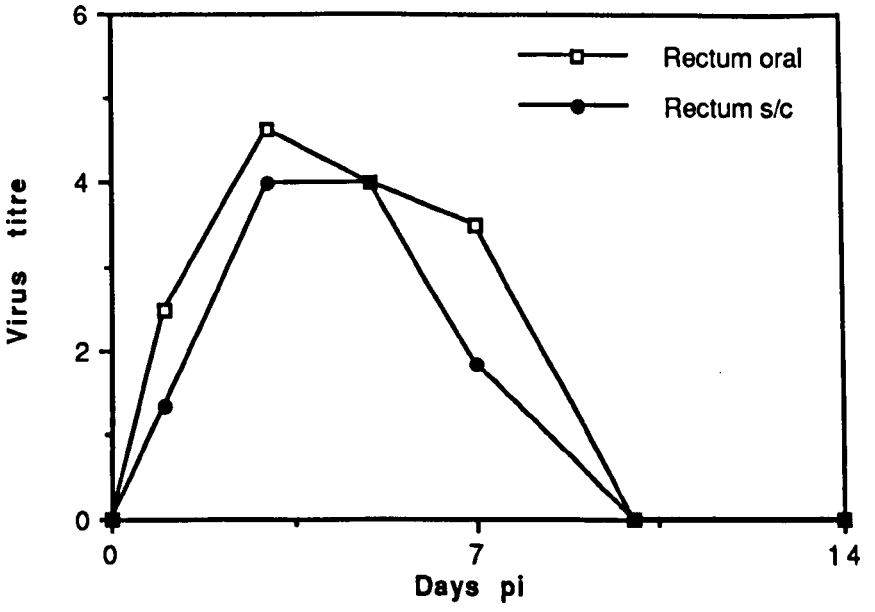

Figure 5. Virus titres (log_{10} $TCID_{50}$ per 50µl) in pooled rectum of chicks infected orally or subcutaneously at day-old with a trypsin-resistant strain R2.

4.3.2 Immunoglobulin assays

Figures 6-9 show reovirus-specific immunoglobulin in pooled intestinal contents and sera of chicks infected orally or subcutaneously when day old.

4.3.2.1 IgA in intestinal contents

There was no detectable virus-specific IgA in the intestinal contents of chicks infected orally or subcutaneously when day-old (Figure 6).

4.3.2.2 IgG in intestinal contents

As with IgA there was no detectable virus-specific IgG in the intestinal contents of chicks infected orally or subcutaneously when day old (Figure 7).

4.3.2.3 IgA in sera

No virus-specific serum IgA was measurable in chicks infected by either route (Figure 8).

4.3.2.4 IgG in sera

Figure 9. shows virus-specific IgG in pooled sera of chicks infected orally or subcutaneously when day old. There was a substantial virus-specific IgG production in chicks infected by both routes. However by day 21 pi, the chicks infected subcutaneously had almost twice the IgG level of those infected orally.

4.3.3 Virus-neutralisation test

No neutralising antibodies could be detected in the intestinal contents because they were toxic to the CELi cells even at a dilution of 1:256. Within 24 hours of incubation, cell sheets were destroyed by the intestinal contents.

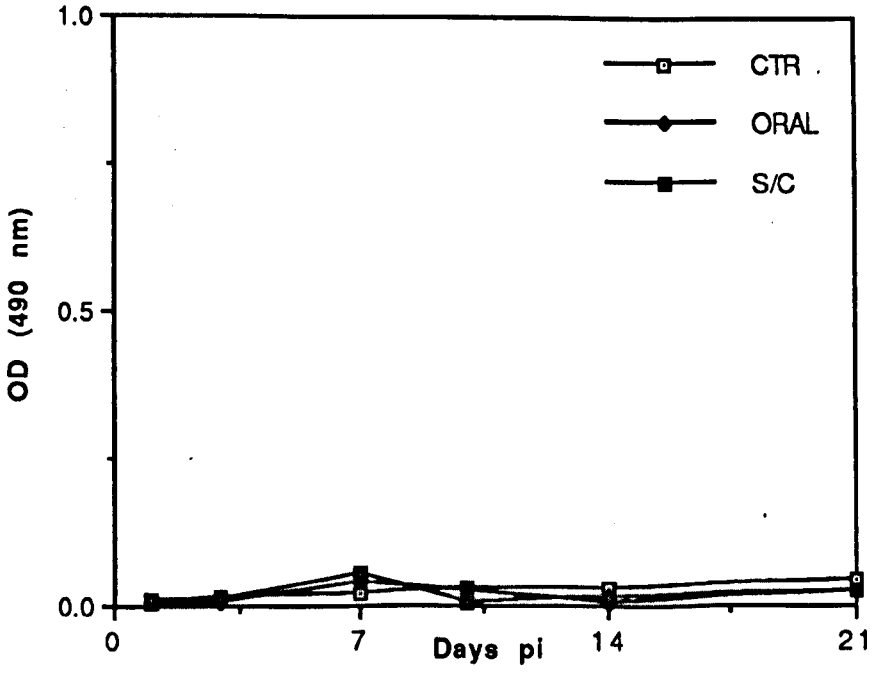

Figure 6. ELISA OD levels of reovirus-specific IgA in pooled intestinal contents from five chickens each infected orally or subcutaneously when day-old.

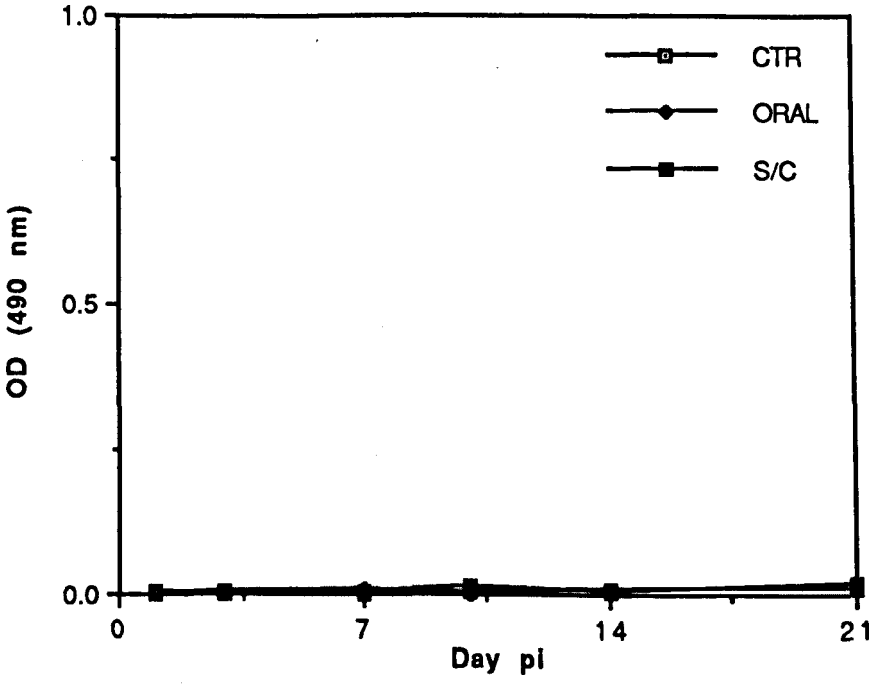

Figure 7. ELISA OD levels of reovirus-specific IgG in pooled intestinal contents from five chickens each infected orally or subcutaneously when day-old.

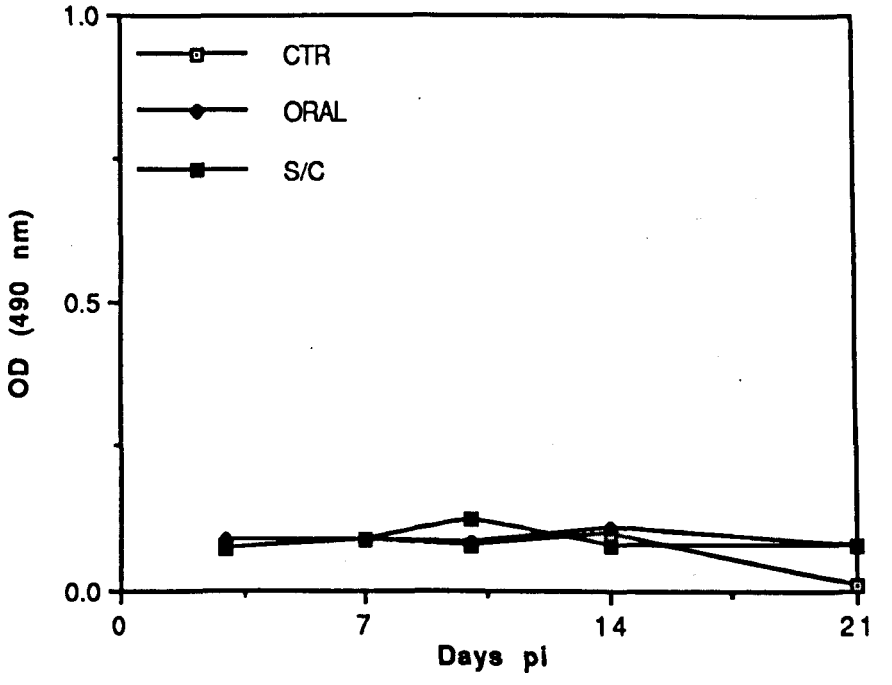

Figure 8. ELISA OD levels of reovirus-specific IgA in pooled sera from five chickens each infected orally or subcutaneously when day-old.

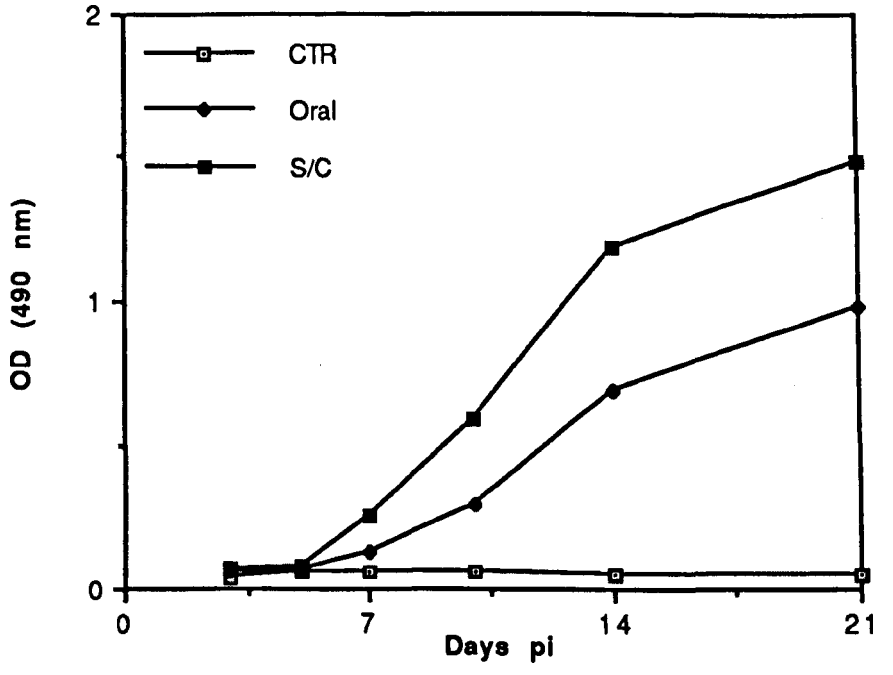

Figure 9. ELISA OD levels of reovirus-specific IgG in pooled sera from five chickens each infected orally or subcutaneously when day-old.

EXPERIMENT 2. COMPARISON OF ORAL AND SUBCUTANEOUS INFECTION OF THREE-WEEK OLD CHICKS

4.4 Materials and methods

This experiment was set up in a similar manner to the first one except that for virus isolation, only the duodenum, jejunum and ileum were used as representative of the intestinal tract and 3 week-old chicks were infected instead of D.O.

4.5 Results

4.5.1 Virus isolation and titration

Virus isolation and titration results are shown in Table 5 and Figures 10 to 12.

On day 1 pi virus was only isolated from the jejunum and ileum of chicks infected orally (Table 5). On days 3 pi virus was isolated from duodenum, jejunum and ileum of the chicks infected orally and subcutaneously. No virus was recovered from any of the gut tissues sampled beyond days 3 pi. Overall, the peak titres in both the oral and subcutaneous routes were similar except for a 2 days delay in onset in the subcutaneously infected birds.

Duodenum

Although the virus titres showed a similar pattern in both routes of infection, the peak titre was higher by 0.63 $\log_{10}$ in the chicks infected orally compared to those infected subcutaneously (Figure 10).

Jejunum

Virus was titratable sooner by 3 days in chicks infected orally than in those infected subcutaneously, but the peak titre was 1.54 log_{10} higher among those subcutaneously infected (Figure 11).

Ileum

Virus titres were again delayed in chicks infected subcutaneously. The peak titre was 0.55 log_{10} higher in chicks infected orally (Figure 12).

Table 5. Virus isolation and titres (log_{10} $TCID_{50}$ per 50µl)from pooled gut samples of chicks infected orally or subcutaneously when 3 weeks old.

	Days pi		
Tissue	1	3	7-21
Duodenum Oral	-	3.93	-
Duodenum S/C	-	3.3	-
Jejunum Oral	3.13	2.93	-
Jejunum S/C	-	4.47	-
Ileum Oral	2.93	3.68	-
Ileum S/C	-	3.13	-

-: no virus isolated.

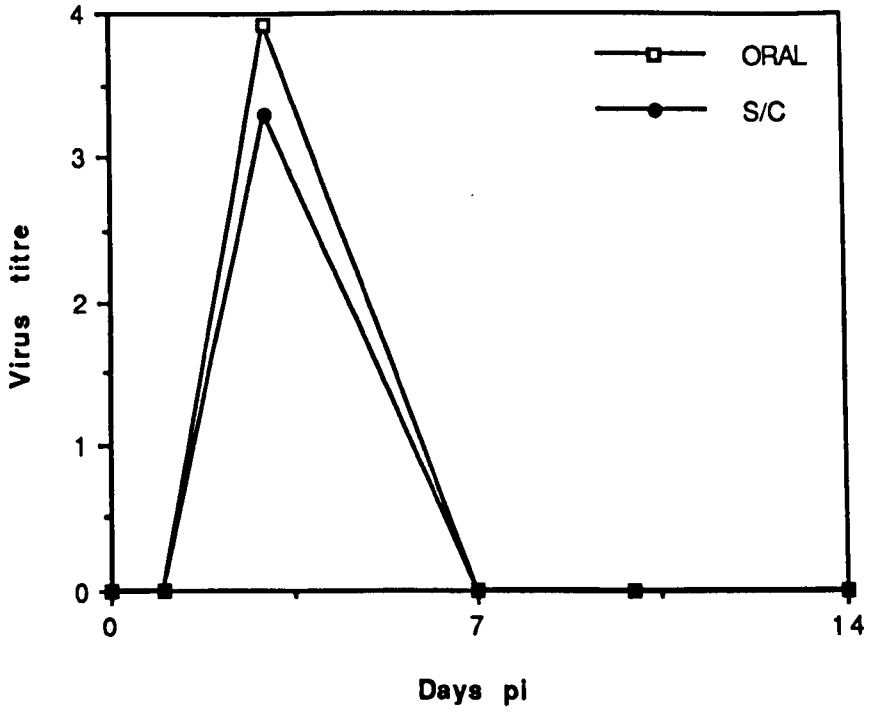

Figure 10. Virus titres (log_{10} $TCID_{50}$ per 50µl) in pooled duodenum of chicks infected orally or subcutaneously when 3 weeks old with a trypsin-resistant strain R2.

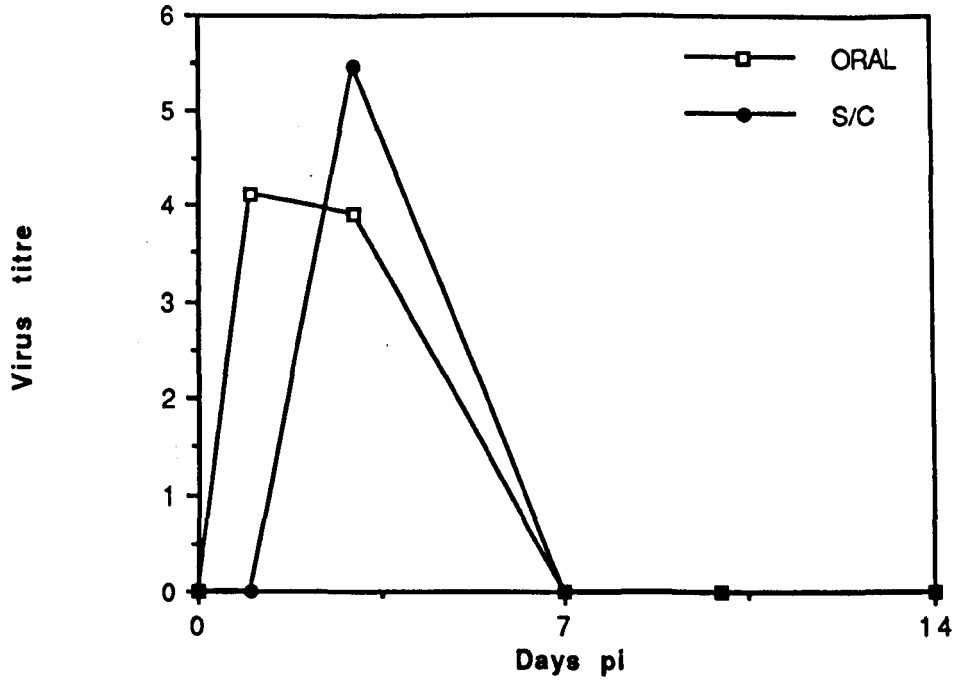

Figure 11. Virus titres (log_{10} $TCID_{50}$ per 50µl) in pooled jejunum of chicks infected orally or subcutaneously when 3 weeks old with a trypsin-resistant strain R2.

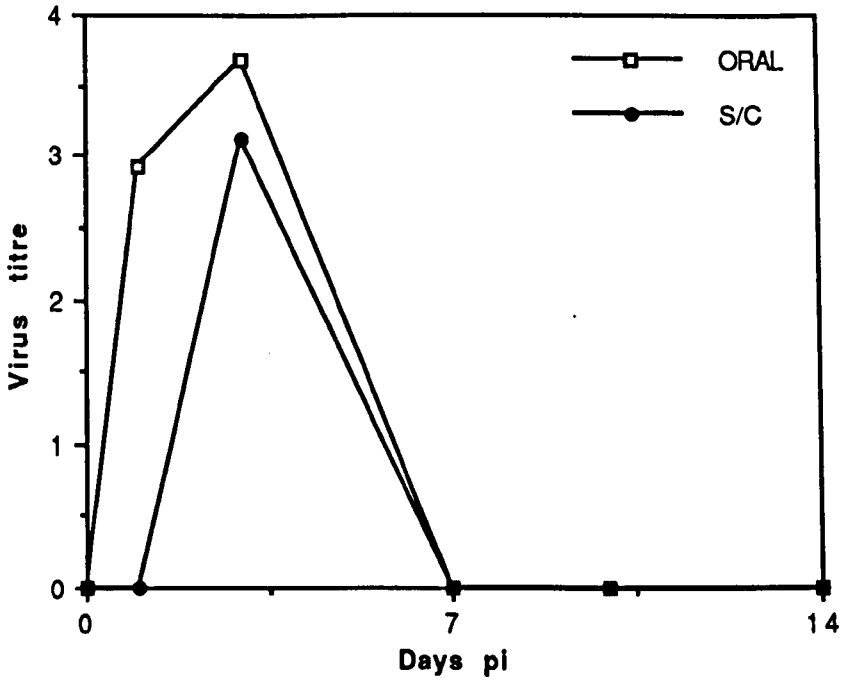

Figure 12. Virus titres (log_{10} $TCID_{50}$ per 50µl) in pooled ileum of chicks infected orally or subcutaneously when 3 weeks old with a trypsin-resistant strain R2.

4.5.2 Immunoglobulin assays

Figures 13-16 show virus-specific immunoglobulin OD values in the intestinal contents and sera of chicks infected when 3 weeks old.

4.5.2.1 IgA in intestinal contents

From days 7 pi reovirus-specific IgA was detected in the gut of chicks infected by either route (Figure 13). However, by end of the experiment the orally infected birds had attained a higher OD level than the subcutaneously infected ones.

4.5.2.2 IgG in intestinal contents

There was no detectable virus-specific IgG in the intestinal contents of chicks infected orally or subcutaneously when 3 weeks old (Figure 14).

4.5.2.3 IgA in sera

No virus-specific IgA was measurable in the serum of chicks infected by either route when 3 weeks old (Figure 15).

4.5.2.4 IgG in sera

Reovirus-specific IgG in sera was measurable by 7 days pi in chicks infected orally or subcutaneously (Figure 16). However, the subcutaneously-infected birds had higher OD levels than the orally-infected ones from days 10-21 pi.

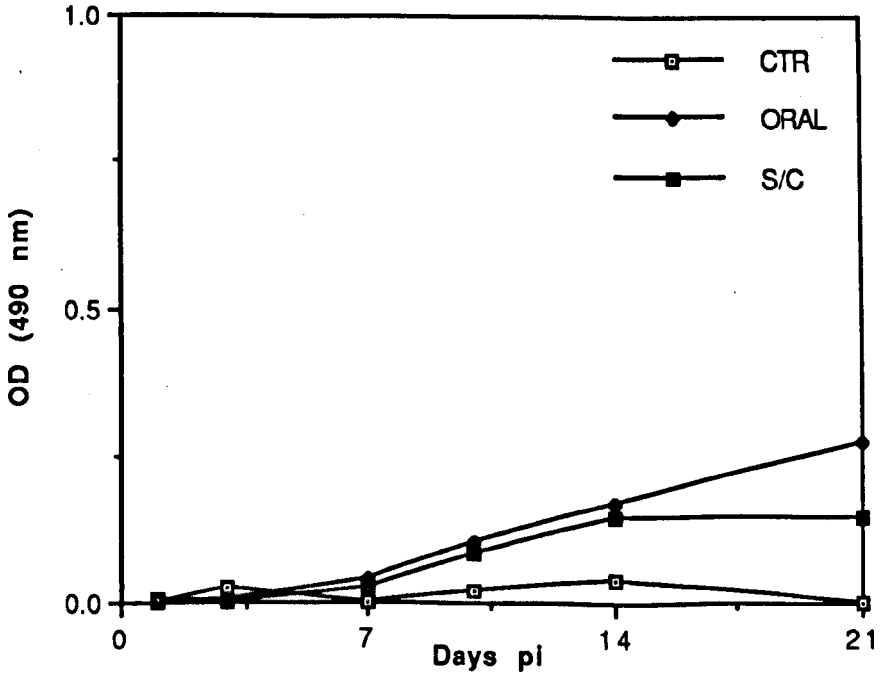

Figure 13. IgA OD levels in pooled intestinal contents of chicks infected orally or subcutaneously when 3 weeks old with a trypsin-resistant strain R2.

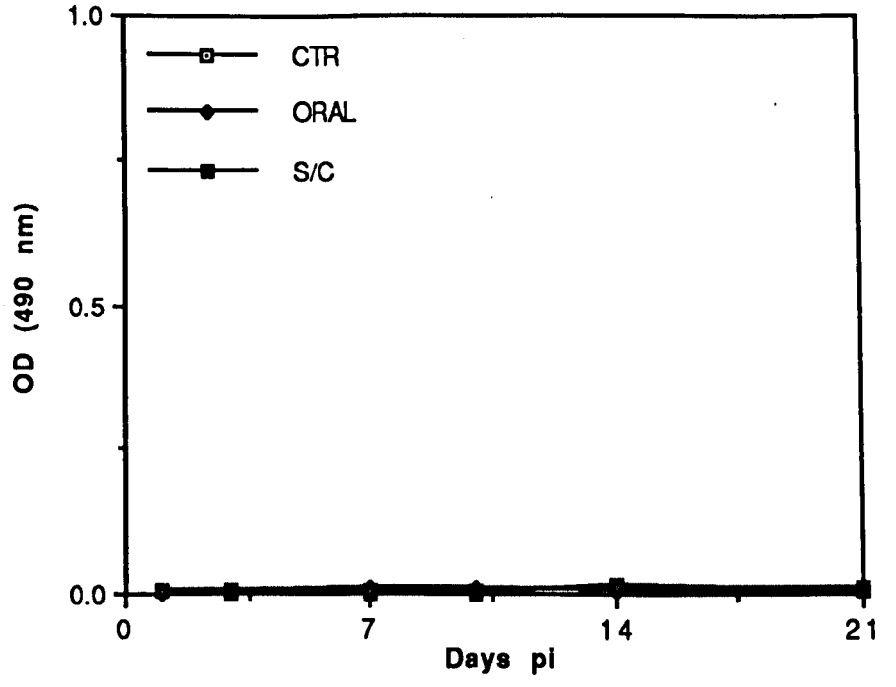

Figure 14. IgG OD levels in pooled intestinal contents of chicks infected orally or subcutaneously when 3 weeks old with a trypsin-resistant strain R2.

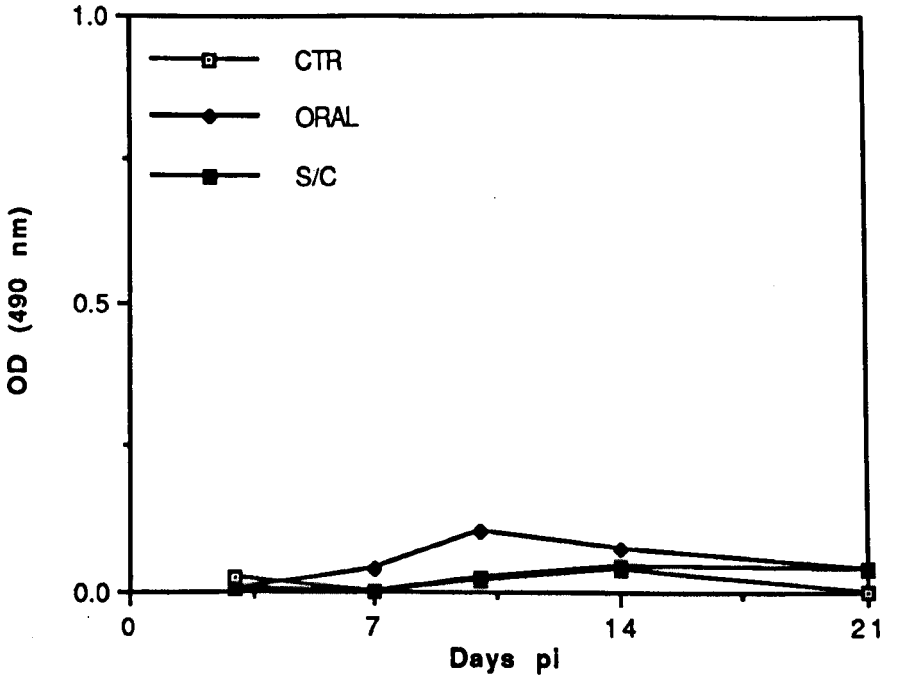

Figure 15. IgA OD levels in pooled sera of chicks infected orally or subcutaneously when 3 weeks old with a trypsin-resistant strain R2.

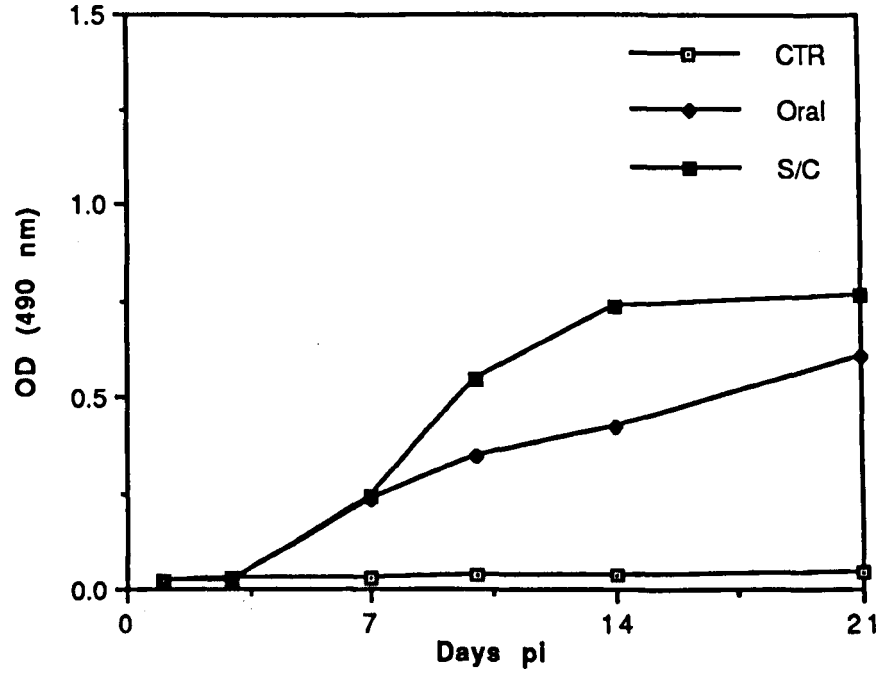

Figure 16. IgG OD levels in pooled sera of chicks infected orally or subcutaneously when 3 weeks old with a trypsin-resistant strain R2.

EXPERIMENT 3. COMPARISON OF ORAL AND SUBCUTANEOUS INFECTION OF SEVEN-DAY OLD CHICKS

4.6 Introduction

In Experiment 1, no reovirus-specific IgA was detectable in the intestinal contents of DO infected chicks, yet in Experiment 2 some IgA was detected in birds infected at three weeks of age. This indicated that the intestinal tract of the older chickens was more responsive to avian reovirus infection by production of IgA. However, 3 weeks of age would not be suitable as a target age to begin to vaccinate chickens against reovirus, because by this time, virulent field virus could have infected the birds and caused damage.

It was therefore considered worthwhile examining a younger age at which the gut would be responsive to reovirus infection, in order to appraise it as a possible vaccination age. One week of age was chosen for this experiment.

4.7 Materials and methods

This experiment was set-up in a similar manner to Experiment 2 except that 7 day-old chicks were used.

4.8 Results

4.8.1 Virus isolation and titration

Virus isolation and titration results of the intestinal tissues from chicks infected orally or subcutaneously when one week old are shown in Table 6 and Figures 17, 18 and 19.

At 3 days pi, virus was recovered from all tissues sampled (Table 6). On day 7 pi virus was recovered from all tissues except the duodenum of orally infected chicks. On day 10 pi virus was isolated from the jejunum of both the subcutaneously and orally infected chicks, while in the ileum, only the subcutaneously infected chicks were positive. The rest of the tissues at 10, 14 and 21 days were negative.

Duodenum

Virus was titratable by day 3 pi in chicks infected orally while virus in those infected subcutaneously was titratable only on day 7 pi (Figure 17). The peak titre of chicks infected orally appeared earlier and was almost 3.0 $\log_{10}$ higher than in those infected subcutaneously.

Jejunum

Both routes of infection had a similar pattern of virus titres (Figure 18), although the chicks infected orally had 1.83 $\log_{10}$ higher peak titres.

Ileum

The virus titre in the chicks infected orally was titratable only on day 3, while that of the chicks infected subcutaneously was only on day 7 pi (Figure 19). The peak titre was almost 3.0 $\log_{10}$ higher in chicks infected orally than in those subcutaneously infected.

Table 6. Virus isolation and titres in gut tissues of chicks infected orally or subcutaneously when one week old with a trypsin-resistant strain R2.

	Days pi			
Tissue	3	7	10	14,21
Duodenum Oral	5.13	-	-	-
Duodenum S/C	+	2.47	-	-
Jejunum Oral	5.3	+	+	-
Jejunum S/C	3.47	+	+	-
Ileum Oral	5.3	+	-	-
Ileum S/C	+	2.68	+	-

+: positive, but titre <0.5 $\log_{10}$; -: no virus isolated

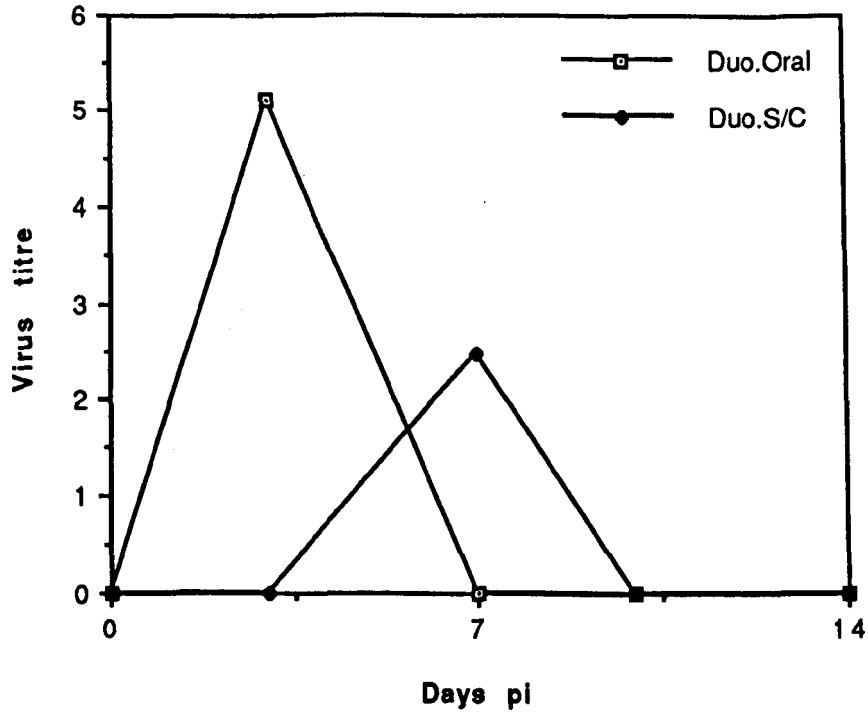

Figure 17. Virus titres from pooled duodenum of five chicks each infected orally or subcutaneously when one week old with a trypsin-resistant strain R2.

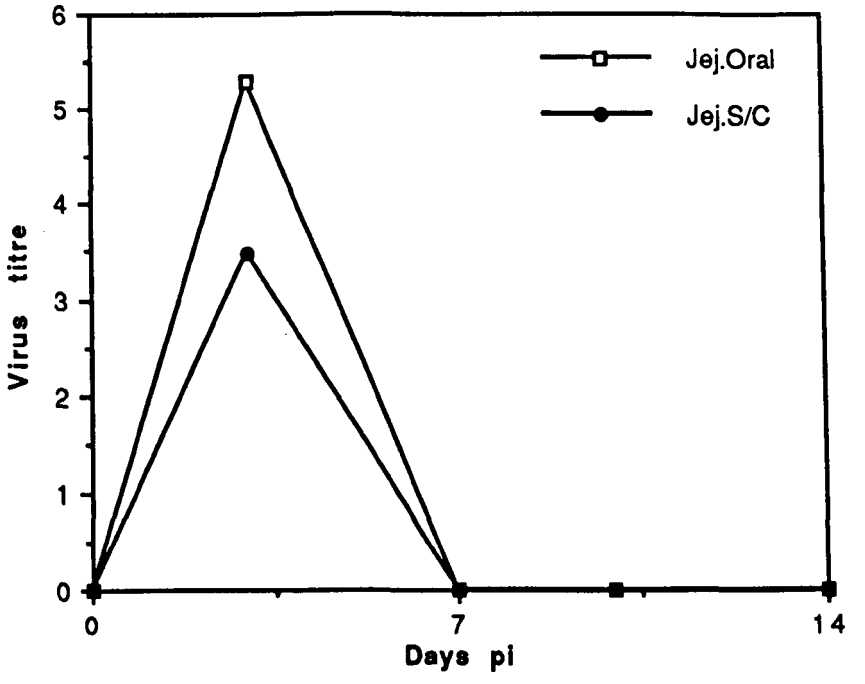

Figure 18. Virus titres from pooled jejunum of five chicks each infected orally or subcutaneously when one week old with a trypsin-resistant strain R2.

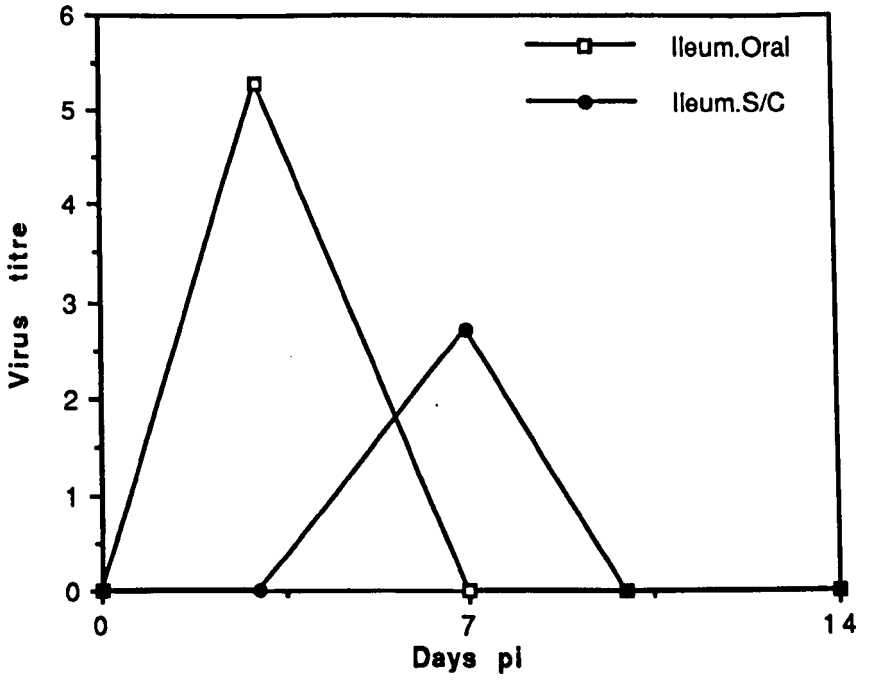

Figure 19. Virus titres from pooled ileum of five chicks each infected orally or subcutaneously when one week old with a trypsin-resistant strain R2.

4.8.2 Immunoglobulin assays

Figures 20-23 show reovirus-specific immunoglobulins in the intestinal contents and sera of chicks infected either orally or subcutaneously when one week old with a trypsin-resistant strain R2.

4.8.2.1 IgA in intestinal contents

A low level of virus-specific IgA was detected in chicks infected orally only (Figure 20), but only after 21 days. The amount was similar to that after infection at 3 weeks (Experiment 2).

4.8.2.2 IgG in intestinal contents

No virus-specific IgG was detected in chicks infected by either route (Figure 21).

4.8.2.3 IgA in sera

No virus-specific IgA was measurable in chicks infected by either route (Figure 22).

4.8.2.4 IgG in sera

Substantial amounts of virus-specific IgG were detected in sera of chicks infected either orally or subcutaneously (Figure 23). By day 14 pi, the OD levels of IgG were higher in the chicks infected orally but both routes had attained similar OD values by day 21 pi.

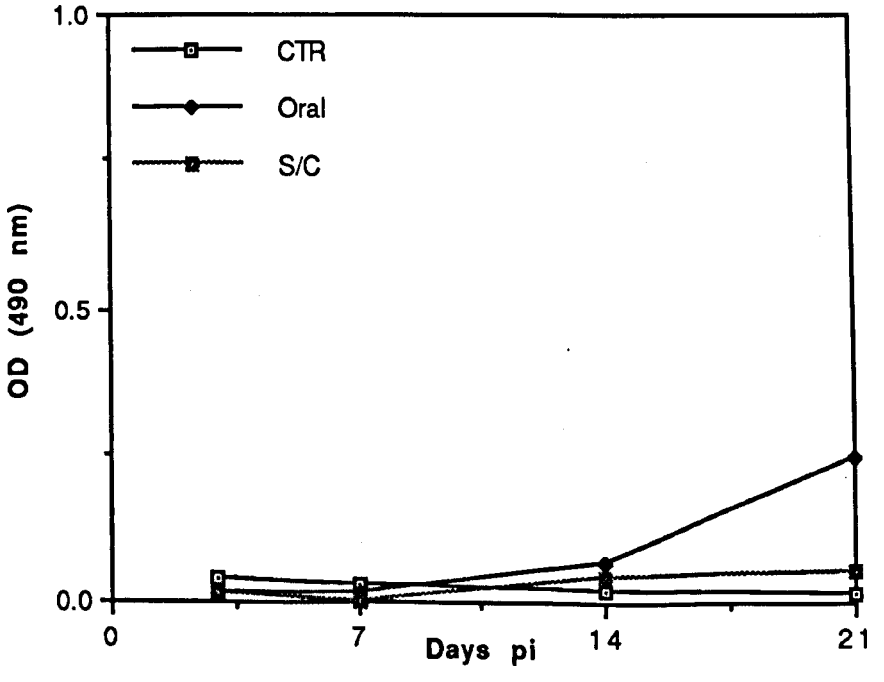

Figure 20. Reovirus-specific IgA OD values of intestinal contents of chicks infected when one week old with a trypsin-resistant strain R2.

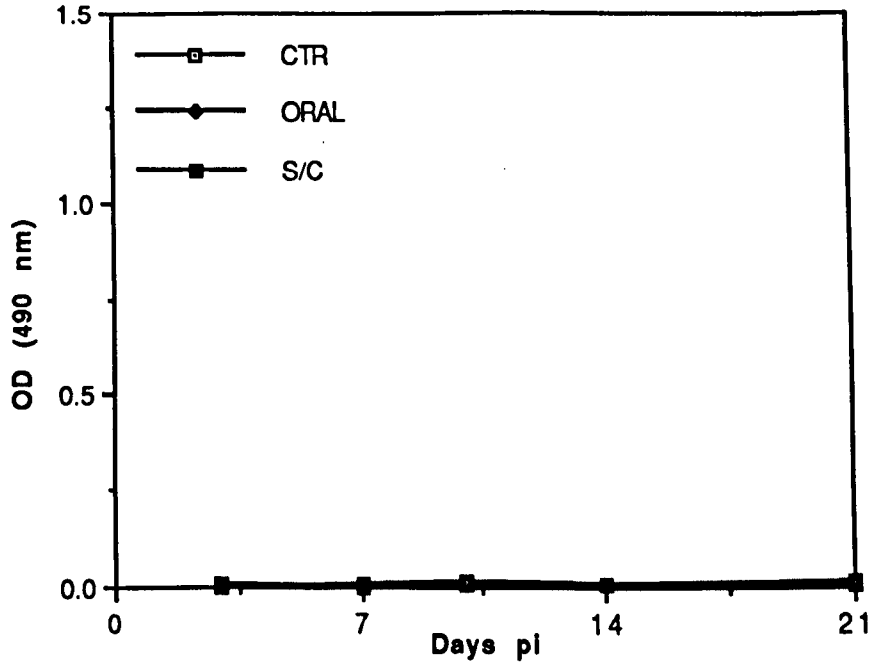

Figure 21. Reovirus-specific IgG OD levels from intestinal contents of chicks infected orally or subcutaneously when one week-old with a trypsin-resistant strain R2.

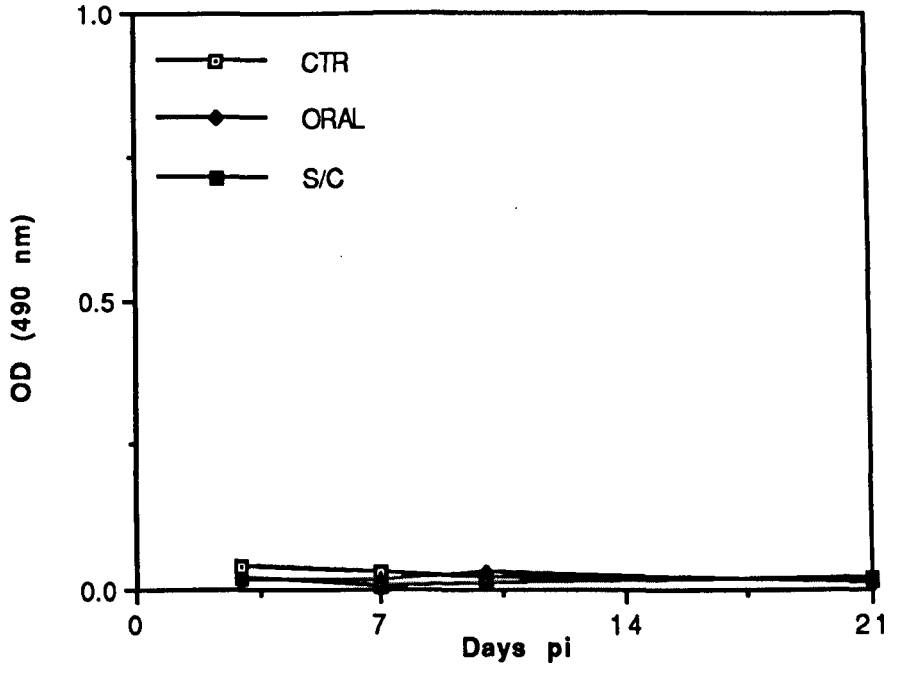

Figure 22. Reovirus-specific IgA OD values of sera from chicks infected orally or subcutaneously when one week-old with a trypsin-resistant strain R2.

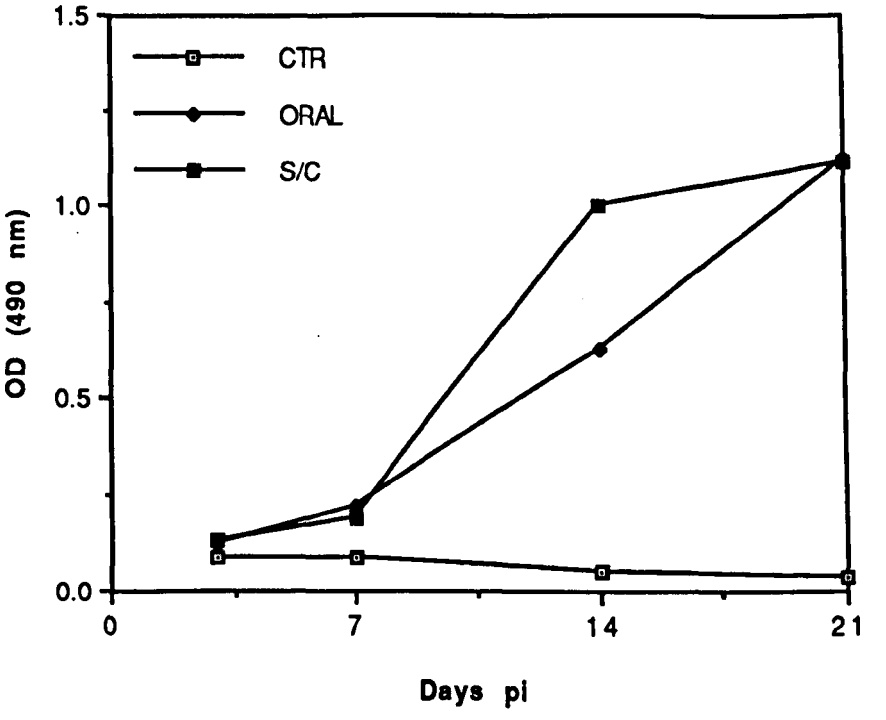

Figure 23. Reovirus-specific IgG OD values of sera from chicks infected orally or subcutaneously when one week-old with a typsin-resistant strain R2.

4.9 Discussion

Part I of this study examined the effect of age and route of infection on the local and systemic antibody responses produced by chicks after infection with a trypsin-resistant reovirus R2. The oral and subcutaneous routes were compared because these are the most common routes of administration of live reovirus vaccines to young chickens.

Although the virus isolation pattern of both the orally and subcutaneously infected D.O. chicks was similar, virus titres in the orally infected birds were some 100-fold higher.

The onset of the virus replication was also slightly delayed in the chicks infected subcutaneously compared to those infected by the oral route. The reason for these differences are presumably because the oral route delivers the virus directly into the gut while by the subcutaneous route, virus takes time via the blood stream before it settles into the intestines for replication. Except in the case of the caecum, where virus titres continued up to day 14 pi, no titratable virus was detected beyond day 7 pi in the intestinal tissues of any chick.

When 3-week old birds were infected, virus was very shortlived in the intestine. It was isolated from the intestinal tissues of chicks infected orally on days 1 and 3 pi, while in those infected subcutaneously, virus was recovered on day 3 pi only.

In the 7-day old infected chicks, virus recovery showed an intermediate situation between D.O and 3 week old infected chicks. Thus virus was recovered up to day 7 pi only and the oral route having a higher titre than the subcutaneous route.

These findings agree with those of Kerr and Olson (1964), Glass et al. (1973), Wood and Thornton (1981) and Jones and Georgiou (1984) who have described evidence of age-linked resistance of chickens to avian reovirus infection.

Although it was not the purpose of this study to examine the role of the caecum, the long and consistent persistence of virus at this site suggests it may have a role in long-term reovirus infections in the chickens.

Following day-old infections, no reovirus-specific IgA was detected in the gut contents of chicks infected by either route. Presumably chicks are insufficiently immunocompetent to produce intestinal IgA at this age. However, virus-specific intestinal IgA was detected in chicks infected orally either when one or 3 week old. Furthermore, those infected orally when one or three week old had higher IgA levels than those infected subcutaneously. The oral route of infection delivers virus directly into the gut and in larger amounts compared to the subcutaneous route. Oral infection caused higher virus titres in the intestine and this was presumably responsible for the higher antibody response.

Young chicks have been suggested as having a less immunocompetent immune system compared to older ones (Jones and Georgiou, 1984). The cause(s) of this immunological unresponsiveness in newly hatched chicks is not clear but lack of helper T-cells (Seto, 1981) and maturation of macrophages (Johnson, 1964; Hirsch et al., 1970) have been suggested. The differences in ability to produce intestinal IgA in different ages of chicks have not been shown before and are likely to be very important. This may explain why reovirus is able to replicate more in the young birds as shown by this study and those of Kerr and Olson (1969), Wood and Thornton (1981), Jones and Georgiou (1984) and Kataria et al. (1988) who have reported increased resistance to infection with age.

Hedge et al., (1982) have reported that development of germinal centres in the chicken gut, which are vital in the immune system are antigen-driven. Young birds therefore are less exposed to environmental antigen and this makes there germinal centres less developed and consequently not as immunocompetent as adults.

Unfortunately, attempts to. detect virus neutralising antibodies in intestinal contents were not successful because the intestinal contents themselves were toxic to the CELi cells used for the test. Thus, it was impossible to say to what extent the intestinal antibodies were antiviral in activity.

No reovirus-specific IgG was detected in the intestinal contents of chicks of any age groups and whichever route was used. This could have been because IgG is prone to proteolytic enzyme degradation in the gut since its does not have secretory component, which protects against intestinal enzymes (Watanabe and Kobayashi, 1974).

Although a proteolytic inhibitor was used in the preparation of intestinal contents in these experiments, some degradation of IgG could have occurred during storage. Porter and Holt (1992), have reported similar findings using an antibody-capture ELISA measuring total IgG in alimentary tract washes. It has also been reported that there is normally little IgG in the gut because its transport from the circulatory sytem is non-specific and the levels increase only after inflammation (Walker and Isselbacher, 1977), when transudation increases. Our results corroborate with those of Myers and Schat (1990) who did not detect any rotavirus-specific intestinal IgG in chickens. Since reoviruses have never been shown to cause extensive inflammation in the gut (Robertson et al., 1986), then IgG would not be expected to transude from circulation to the intestines.

No reovirus-specific IgA was detected in sera of chicks infected by either route in whatever age group. The reason for the absence of serum IgA is not clear. It has been reported that there are low levels of serum IgA compared to the levels in external secretions. Myers and Schat (1990) detected avian rotavirus-specific serum IgA for a much shorter time compared to intestinal IgA. In humans, a seroprevalence study of anti-reovirus serum IgA showed similar results (Selb and Weber, 1994).

Serum IgA is mainly monomeric while the form in external secretions is dimeric or tetrameric (Mestecky and McGhee, 1987). It is probable that there are more epitopes in the intestinal IgA because of their dimeric or tetrameric nature and hence an increased binding affinity in the ELISA assay. The superiority of secretory IgA antibody over serum IgA in functions such as agglutinations or precipitations in mammals is well documented (Heremans 1974). Furthermore, it has been reported that both systemic immunization and oral ingestion of antigens of short duration usually does not lead to the induction of a preferential serum IgA response (Mestecky and McGhee, 1987). A further possibility is that the high-affinity IgG antibody may have obscured measurement of IgA antibody (Selb and Weber, 1994).

Virus-specific serum IgG was detectable in chicks inoculated by either route and in all age groups. However, those infected subcutaneously consistently responded with higher levels of IgG than those infected orally. This is the opposite of the situation with virus-specific intestinal IgA in that the chicks infected orally responded with a higher IgA levels. Similarly, the reason could be the introduction of virus almost directly into the blood system where plasma cells in organs such as the spleen (Firth, 1977), would have a higher chance of producing antibody.

It is not clear whether these higher virus-specific IgG levels in sera can be correlated with a higher degree of protection if this was a reovirus vaccine. However, for protection against enteric pathogens it is vital that the gut-associated lymphoid tissue is exposed to locally available antigen in order to produce local antibodies and in this regard the oral route would appear to be more appropriate.

Perhaps It would seem that the value of circulating IgG could be to minimise or prevent spread of virus to target sites, especially the hock joints, once the bird is infected.

The next part of this chapter examines the effect of using a trypsin-senstive reovirus.

PART II

INFECTION WITH A TRYPSIN-SENSITIVE VIRUS

4.10 Introduction

Following natural infection of chickens with avian reovirus, there is an initial replication of virus primarily in the mucosa of the digestive and respiratory tracts (Menendez et al., 1975; Ellis et al., 1983). This is followed by a viraemia resulting in virus spread to various tissues (Menendez et al., 1975; Ellis et al., 1983: Kibenge et al., 1985), after which virus settles in the joints where it may persist for long periods (Macdonald et al., 1978; Marquardt et al., 1983; Jones and Kibenge, 1984).

Although previous work had considered all avian reoviruses to be trypsin-resistant (Kawamura et al., 1965; Petek et al., 1967; Jones et al., 1975), Al-Afaleq and Jones (1991) isolated a trypsin-sensitive avian reovirus from the hock of turkey. Furthermore, Jones et al. (1996) recently examined 21 strains of avian reovirus and found that one third were trypsin-sensitive. The pathogenicity of such trypsin-sensitive strains has been shown to be reduced in the intestinal tract (Al-Afaleq and Jones, 1991; Jones et al., 1994) and the development of tenosynovitis reduced (Drastini et al., 1994).

Since trypsin is one of several digestive enzymes, it may not only affect the infectivity, virulence, and persistence, of such a strain, but it could also affect the chicken's capacity to induce an effective immune response if the oral route of infection is used.

Many avian reovirus vaccines are related to strain S1133 which has been found to be trypsin-sensitive (Jones et al., 1996). Such vaccines are therefore likely be relatively poor perfomers if given by the oral route.

The purpose of this study was to compare the antibody responses of chickens given a trypsin-resistant reovirus by the oral or subcutaneous routes in day-old and one-week old chicks.

EXPERIMENT 1. COMPARISON OF ORAL AND SUBCUTANEOUS INFECTION OF DAY-OLD CHICKS

4.11 Materials and methods

4.11.1 Virus

The trypsin-sensitive strain TR1, originally isolated from the hock of a turkey with arthritis and antigenically similar to R2 (Al-Afaleq and Jones, 1990) was used.

4.11.2 Experimental chickens

All chicks were housed and maintained from hatch to death as described in Chapter 3. SPF one-day old were used in Experiment 1, and seven-day old chicks in Experiment 2.

4.11.3 Experimental design

Seventy five day old SPF chicks were divided into three groups of twenty-five birds each. One group was orally infected with 0.3 ml of M199 media containing 3.5 $\log_{10}$ $TCID_{50}$ per 50µl of TR1 while the other was infected with the same dose of virus subcutaneously. These two infected groups were kept in separate pens. The third group was inoculated with M199 media only and kept separately as a control.

4.11.4 Sampling, virus isolation and immunoglobulin assays

On days 3, 5, 7, 14 and 21 pi five birds were taken from each of the groups for sampling. The birds were first bled and thereafter humanely killed. They were aseptically opened and the intestinal tract removed. Samples for virus isolation and immunoglobulin assays were perfomed as described in Part I.

4.12 Results

4.12.1 Virus isolation

No virus was isolated at any time from the intestinal tissues of either the orally or subcutaneously infected chicks.

4.12.2 Immunoglobulin assays

4.12.2.1 IgA and IgG in intestinal contents

Virus-specific IgA and IgG were not detected in the intestinal contents of chicks infected by either route used. (Figures 24 and 25).

4.12.2.2 IgA in sera

No virus-specific serum IgA was detected in chicks infected either orally or subcutaneously when DO with the tryspin-sensitive reovirus TR1 (Figure 26).

4.12.2.3 IgG in sera

Virus-specific IgG was detected by day 14 pi in the sera of chicks infected orally or subcutaneously. The latter chicks had OD levels which were twice as high as those of the orally-infected birds by day 21 (Figure 27).

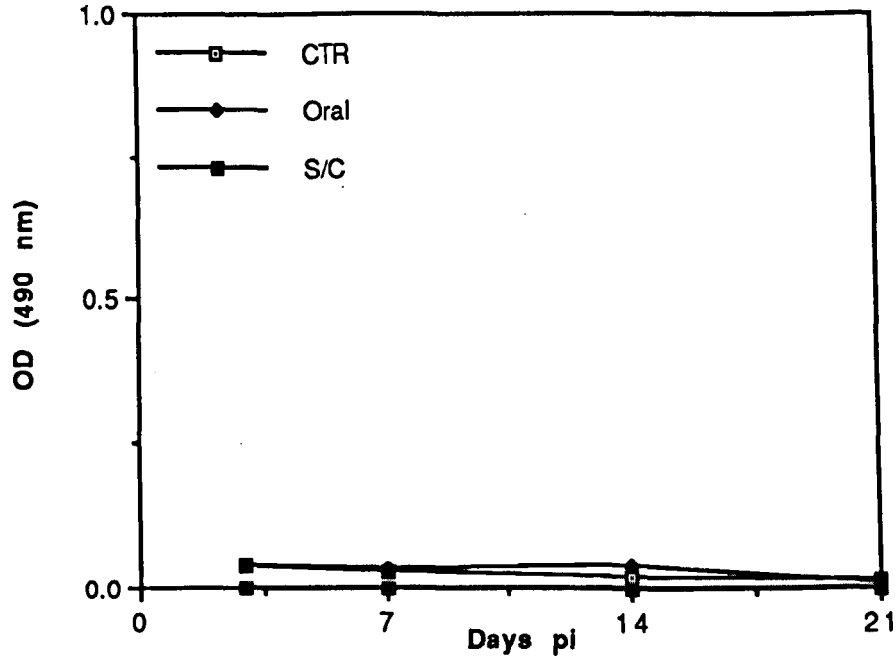

Figure 24. Reovirus-specific IgA OD values of intestinal contents from DO chicks infected orally or subcutaneously with a trypsin-sensitive strain TR1.

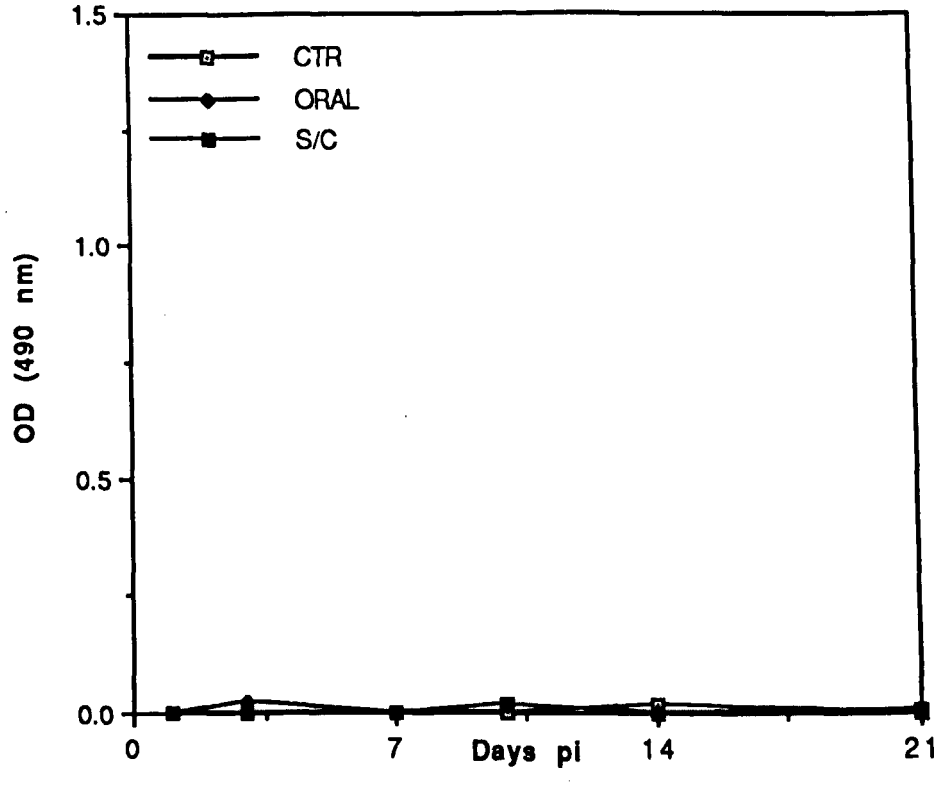

Figure 25. Reovirus-specific IgG OD values of intestinal contents from DO chicks infected orally or subcutaneously with a trypsin-sensitive strain TR1.

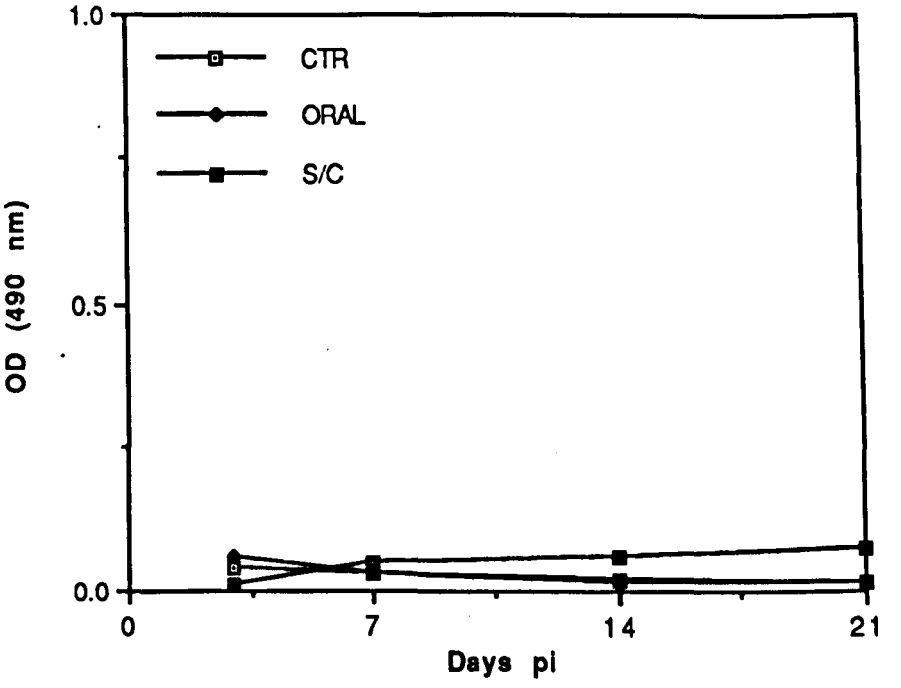

Figure 23. Reovirus-specific IgA OD values in sera from DO chicks infected orally or subcutaneously with a trypsin-sensitive strain TR1.

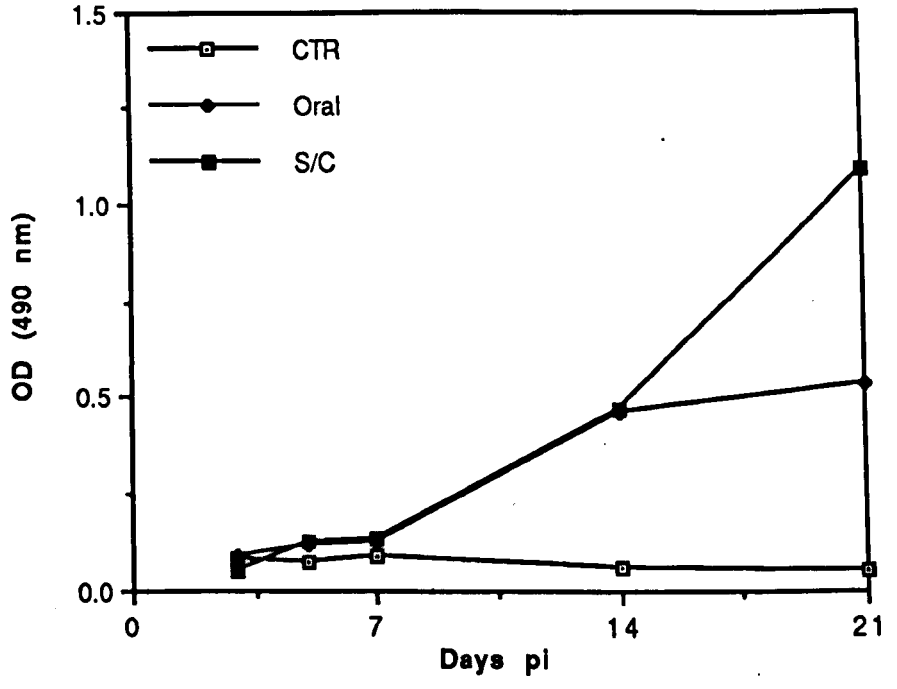

Figure 27. Reovirus-specific IgG OD values in sera from DO chicks infected orally or subcutaneously with a trypsin-sensitive strain TR1.

EXPERIMENT 2. COMPARISON OF ORAL AND SUBCUTANEOUS INFECTION OF SEVEN-DAY OLD CHICKS

4.13 Materials and methods

These were similar to those used in Experiment 1, except that seven-day old chicks were infected instead of DO.

Since no virus was isolated from the intestinal tissues of any of the infected chicks in the previous experiment, some tissues other than those from the gut were also sampled in this experiment. Thus the bursa, kidney, liver, lung and spleen were collected for virus isolation in addition to duodenum, jejunum and ileum.

4.14 Results

4.14.1 Virus isolation

No virus was isolated from the intestinal tissues of any of the chicks infected orally or subcutaneously. Similarly, no virus was isolated from the liver, bursa, kidney or spleen. However, virus was isolated from the lung on day 3 pi in the orally-infected group.

4.14.2 Immunoglobulin assays

4.14.2.1 IgA in intestinal contents

No virus-specific IgA was detected in the intestinal contents of chicks infected by either of the routes (Figure 28).

4.14.2.2 IgG in intestinal contents

No virus-specific IgG was detected in the chicks infected by either (Figure 29).

4.14.2.3 IgA in sera

No virus-specific serum IgA was detected in chicks infected by either of the routes (Figure 30).

4.14.2.4 IgG in sera

Virus-specific IgG was detected in sera of chicks infected by both routes (Figure 31). The subcutaneous route of infection induced higher OD levels than the oral route.

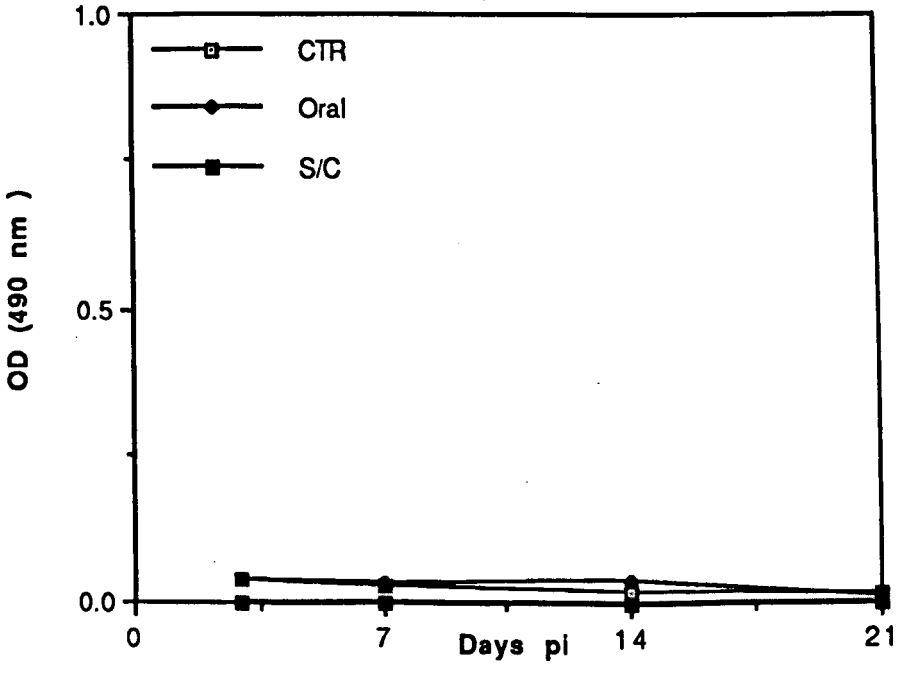

Figure 28. Reovirus-specific IgA OD values of intestinal contents from 7-day old chicks infected orally or subcutaneously with TR1.

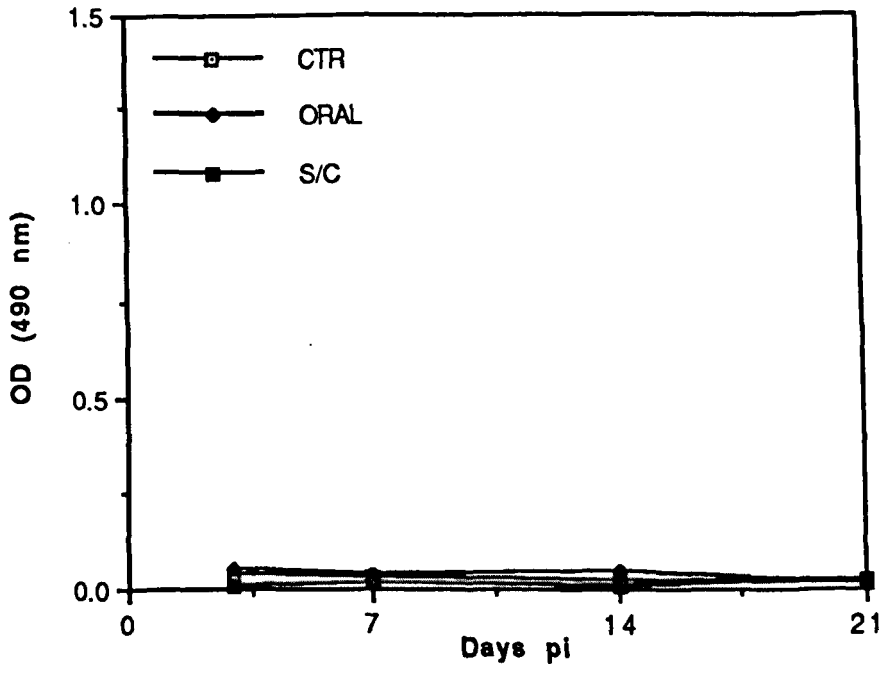

Figure 29. Reovirus-specific IgG OD values of intestinal contents from 7-day old chicks infected orally or subcutaneously with TR1.

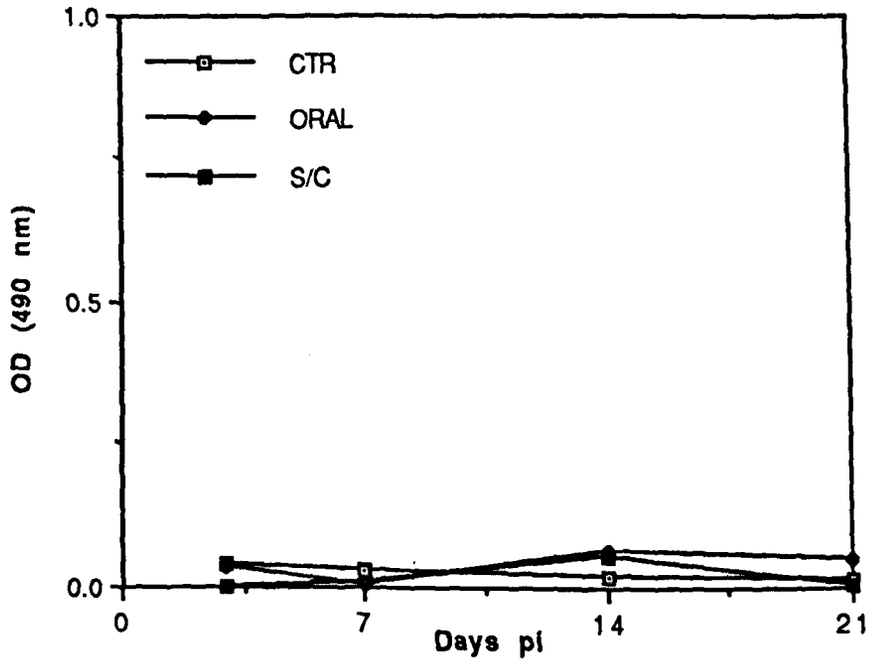

Figure 30. Reovirus-specific IgA OD values in sera from 7-day old chicks infected orally or subcutaneously with TR1.

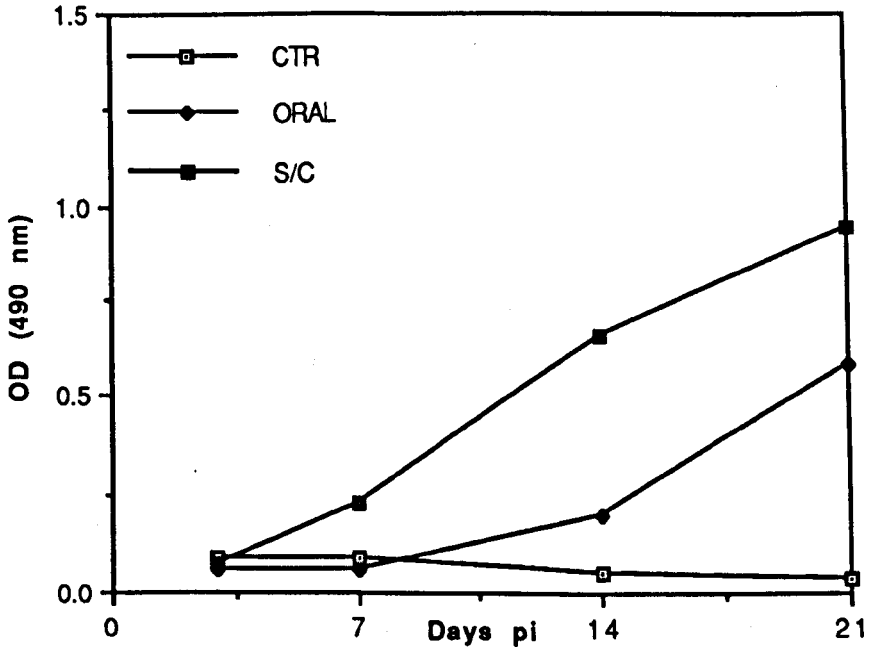

Figure 31. Reovirus-specific IgG OD values in sera from 7-day old chicks infected orally or subcutaneously with TR1.

4.15 Discussion

Recent studies have established that some avian reoviruses are trypsin-sensitive and this characteristic affects their pathogenesis (Al Afaleq and Jones, 1991; Jones et al 1996). This feature is also likely to affect their epidemiology through restricting replication in the gut, a tissue which has for long been considered vital for the spread of reovirus (Menendez et al., 1975a; Ellis et al., 1983; Kibenge et al., 1985; Jones et al., 1989).

This study was directed at defining how trypsin sensitivity may affect the capacity of TR1 to induce local or systemic immune responses in DO or one week old chicks, when infected orally or subcutaneously.

TR1 was never reisolated from the intestinal tissues of the chicks infected either orally or subcutaneously and in either of age groups. Having failed to isolate the virus in the gut tissues in the first experiment, an attempt was made to isolate it from some non-enteric tissues (lung, spleen, kidney, liver and bursa) as well as in the gut in chicks infected orally or subcutaneously when one week old. No virus was isolated from the non-gut tissues except from the lungs of chicks infected orally and the isolation was on day 3 pi and in negligible (non-titratable) amounts. It is likely that virus administered via the oral route could have drained into the nasal cavity through the choanal cleft or entered the respiratory tract by aspiration. Jones et al. (1994) have shown that after intranasal infection of chicks with TR1, there were high virus titres in the lungs and they suggested that the respiratory route could be of importance for spread of these trypsin-sensitive reoviruses.

The lack of isolation of TR1 from non-enteric tissues (except the lungs), after subcutaneous infection is surprising and may lead us to reaffirm that replication of reovirus in the gut plays a vital role in the dessimination of this virus to other parts of the body. However, this would raise the question of how the typsin-sensitive viruses maintain themselves in the poultry population.

No virus-specific intestinal IgA was detected in the chicks infected by either route or age group. Although the younger age of the chicks used could have contributed to this finding, lack of replication in the gut is likely to be the main reason in the 7 day old birds. This is because virus-specific intestinal IgA was detected in chicks infected orally when one week old in the previous experiment (Part I, Experiment 3). Lack of replication by TR1 in the gut, or its limited replication as reported by Al-Afaleq and Jones (1990), and Jones et al. (1994, 1996) confirms the fact that exposure of TR1 to intestinal trypsin and perhaps other enzymes is a limiting factor to the replication of this strain of virus.

Virus-specific serum IgG was detected in chicks infected by either route and in both age groups. This suggests that even without apparent replication even in the bursa or spleen, TR1 can induce substantial amounts of systemic IgG in chicks. However, one vital source of plasma cells, bone marrow, was not sampled in this study.

The levels of virus-specific serum IgG induced in chicks infected orally were identical as well as the levels in the subcutaneously-infected groups. However, virus-specific serum IgG was higher in chicks infected subcutaneously than those infected orally. This result mimics that found in chicks infected either orally or subcutaneously with the trypsin-resistant strain R2 (Part I).

These results shows that there is need for more work to be done with these trypsin-sensitive strains of reovirus in order to establish where they do replicate or exert the immunopotentiation. Because a constant dose was used in this work, the efficacy of a higher dose on the replication of TR1 can not be ruled out. Jones et al. (1994) have shown that a high dose is necessary for the trypsin-sensitive virus to replicate in the jejunum.

In conclusion, the findings in this chapter demonstrate the importance of considering age, route and trypsin sensitivity of the strain of virus when testing for the efficacy of candidate reovirus vaccines. Thus, if the aim of a vaccination programme is to induce protection via the production of intestinal local immunity, then a trypsin-resistant strain of reovirus given at about one-week of age might be the ideal vaccine. However, if sytemic protection is the targeted response, then either a trypsin-sensitive or a trypsin-resistant strain can be used as a vaccine. Considering the fact that chicks are infected soon after hatch and usually via faecal-oral route, a trypsin-resistant strain has the advantage in that it can induce both local and systemic responses when given within a week of hatch.

CHAPTER 5

EFFECT OF B-CELL IMMUNOSUPPRESSION ON INTESTINAL AND SYSTEMIC IMMUNE RESPONSES OF CHICKS INFECTED WITH AVIAN REOVIRUS

CONTENTS PAGE

CHAPTER 5

EFFECT OF B-CELL IMMUNOSUPPRESSION ON INTESTINAL AND SYSTEMIC IMMUNE RESPONSES OF CHICKS INFECTED WITH AVIAN REOVIRUS

5.1 Introduction

The immune status of chickens has been shown to have an influence on the outcome of reovirus infection. Chickens infected with reovirus and infectious bursal disease virus, a known immunosuppressant in chickens, had lower antibody titres to reovirus compared to birds infected with reovirus only (Springer et al., 1983; Moradian et al., 1990). Futhermore, Kibenge et al. (1987) demonstrated that neonatal chicks treated with cyclophosphamide, a B-cell suppressor had a markedly increased duration of cloacal virus shedding and most of the birds lacked reovirus neutralising antibodies.

The effects of B-cell suppression on humoral immunity in chickens have been evaluated on virus neutralising antibodies (Kibenge et al., 1987; Okoye and Uzoukwu, 1990), precipitating antibodies (Lerner et al., 1971; Bryant et al., 1973; Toivanen and Toivanen, 1973; Hirota et al., 1976; Kibenge et al., 1987; Okoye et al., 1992) immunofluorescent antibodies (Yuasa et al., 1988) or total serum immunoglobulins (IgA, IgG or IgM) (Lerner et al., 1971; Martin and Leslie, 1973; Perey and Bienenstock, 1973; Hirota et al., 1976) as a measure of the responses. There are few reports where class-specific immunoglobulins have been monitored to assess B-cell suppression in chicken after infection. An example is the assessement of anticoccidial IgG in sera and IgA in bile of bursectomised chicks after infection with coccidia (Lillehoj, 1987).

It is well documented that the intestinal tract is one of the predilection sites for avian reoviruses (Menendez et al., 1975; Ellis et al., 1983; Jones et al., 1986). The presence of virus in the gut is, however, relatively short-lived compared to other parts of the body and in particular, the joints (Olson and Kerr, 1967; Jones and Onunkwo, 1978; Marquardt et al., 1983; Al-Afaleq and Jones, 1994). The rapid clearance of virus from the gut has been attributed by Kibenge et al. (1987) in part to the humoral immune mechanisms.

The role of the mucosal immune response to reovirus infection has not had the attention it deserves, and in Chapter 4 it has been shown that locally-produced reovirus-specific IgA may take part in the clearance of virus from the intestinal tract.

The objective of these experiments was to establish what effects abrogation of humoral immune mechanism has on persistance of virus in different regions of the intestine and the local immune responses in the gut after reovirus infection. The effect of route of administration of reovirus and age of chicks when immunossupressed were also examined.

EXPERIMENT 1. EFFECT OF B-CELL IMMUNOSUPPRESSION ON INTESTINAL AND SYSTEMIC IMMUNE RESPONSES OF CHICKS INFECTED WHEN DAY-OLD WITH AVIAN REOVIRUS STRAIN R2

Day-old chicks previously treated with testosterone-propionate on the third day of embryonation, were infected orally or subcutaneously with reovirus strain R2. The mucosal immune response in the gut and systemic response were examined by measurement of reovirus-specific IgA and IgG. in intestinal contents and sera respectively.

5.2 Materials and methods

5.2.1 Chicks

DO old hybrid SPF chicks were used. They were given water and food *ad libitum*. They were kept in strict isolation as described in Chapter 3.

5.2.2 Virus

Avian reovirus strain R2 was used in this experiment and details have been described in Chapter 3.

5.2.3 Embryonal bursectomy

The method used was a modification of the hormonal bursectomy described by Glick and Sadler (1961) and May and Glick (1964). A similar procedure has recently been used by Okoye and Uzoukwu (1990). Eggs were dipped for five seconds in a solution of methyltestosterone (Sigma) (1g in 100ml of absolute alcohol) at room temperature on the third day of incubation. All treated chicks were subsquently proved to have no bursa of Fabricius at the various sampling times.

5.2.4 Experimental design

One hundred and twenty day-old bursectomised (Bx) chicks were used. They were divided into three groups of 40 chicks each and kept in separate isolation rooms. One group was inoculated orally by oesophageal cannulation with 0.3 ml of M199 medium containing 3.2 $\log_{10}$ $TCID_{50}$ per 50µl of R2. A second group was inoculated with a similar quantity of the virus but subcutaneously. The third group was left uninfected and kept as controls.

Five chicks from each group were bled and humanely killed on days 1, 3, 5, 7, 10, 14, 21 and 28 pi. The intestinal tract was aseptically removed and various tissues were taken for virus isolation and local antibody estimation.

5.2.5 Intestinal tract sampling, intestinal content treatment and virus isolation and titration

These were done as stated in Chapter 4.

5.2.6 Test for B-cell function

***5.2.6.1 Brucella abortus* antigen.** Killed *Brucella abortus* antigen was obtained commercially (Murex diagnostics Ltd, Dartford, UK). In order to remove preservatives and stains, the antigen was washed three times in PBS by centrifugation at 1500g and resuspended in PBS to its original volume.

***5.2.6.2 Brucella abortus* antigen administration and sample collection.** Each chick was given 0.2 ml of the undiluted *Brucella abortus* antigen by intra-peritoneal inoculation. Fifteen day-old chicks were divided into three groups of five each. The first group consisted of Bx and then brucella-inoculated chicks, the second were non-Bx but brucella-inoculated, while the third were non-bursectomised and non-inoculated, to act as controls. Blood samples were were obtained from the wing vein on 3, 7, 10 and

14 days pi. and allowed to clot. Sera were separated, inactivated at 56^0C for 30 minutes and kept at -20^0C until agglutination assays were run.

5.2.6.3 Titration of *Brucella abortus* antibodies. Two-fold dilutions of sera in PBS starting at 1:4 , in 50µl volumes were made in round-bottomed microtitration plates (Bibby Sterilin Ltd, Stone, UK). An equal quantity of *Brucella abortus* antigen diluted 1:10 in PBS was added to each well. The plates were then shaken for one minute and incubated for 24 hours at room temperature in a humidified chamber (Munns and Lamont, 1991). The agglutination titre was expressed as the $\log_2$ of the reciprocal of the highest dilutions in which complete agglutination occurred.

5.3 Results

No mortalities occured following the method of Bx used.

5.3.1 Brucella agglutination assays

Mean titres from 5 chicks are shown in Figure 1. There was very low agglutination ($\log_2$ 2) in sera from bursectomised chicks after inoculation with brucella antigen. The sera from the non-bursectomised chicks, showed agglutination of 4.6 $\log_2$ on day 3 pi and reached a peak of 10.6 $\log_2$ on day 10 pi. These results indicated that the bursectomy was effective.

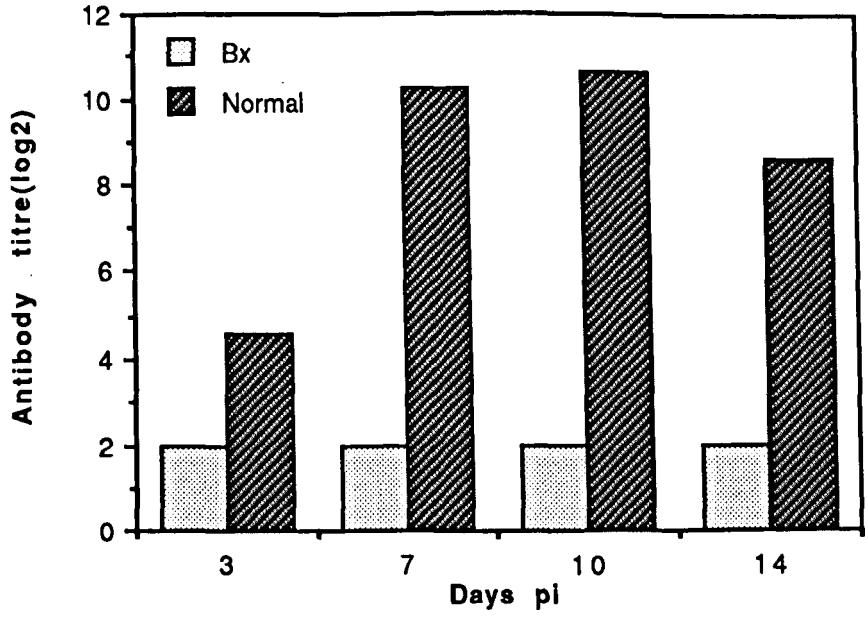

Figure 1. Mean antibody titres (log_2) to *Brucella abortus* antigen in sera of bursectomised (Bx) or non-bursectomised chicks after inoculation with *Brucella abortus* when day-old.

5.3.2 Virus isolation

Virus isolation from intestinal tissues of non-imunosuppressed (normal) chicks infected orally or subcutaneously when day-old with reovirus strain R2 are shown in Tables 1 and 3 while those from the immunosuppressed (Bx) chicks are shown in Tables 2 and 4.

Tissue isolation indices were established as a measure of tissue invasiveness by the virus during the period of sampling. Three passages were attempted before a tissue was declared negative. Thus a score of 3 was given when virus was recovered on first passage, 2 on second passage, 1 on third passage and 0 when the tissue was negative. The isolation index was calculated as the total of the isolation scores during the course of the experiment.

In the Bx groups the experiment was extended for up to 28 days pi in anticipation of prolonged virus isolation as a result of immunosuppression.

The results show no striking differences between the indices of the Bx and normal chickens in each of the orally or subcutaneously infected groups. However, a comparison of the normal and Bx chicks infected subcutaneously (Tables 3 and 4), show that the onset of virus recovery was delayed in the Bx chicks by about four days. There was a similarity in the total isolations from chicks infected by the oral route and also a similarity in those infected by the subcutaneous route, irrespective of whether they were immunosuppressed or not.

Among the chicks infected orally (Tables 1 and 2), the caecum yielded virus most consistently and had the highest isolation index in both the normal and Bx groups.

Table 1. Virus isolation indices of pooled (n=5) intestinal regions of normal chicks infected orally when day-old with reovirus strain R2.

Dpi Tissue	1	3	5	7	10	14	21	Index
Duo	3	3	3	3	2	0	1	15
Jej	3	3	3	3	0	0	0	12
Ileum	3	3	3	2	0	3	0	14
Caecum	2	3	3	3	3	3	3	20
Rectum	3	3	3	3	2	3	0	17

Total isolation indices 78

Table 2. Virus isolation indices of pooled (n=5) intestinal regions of Bx chicks infected orally when day-old with reovirus strain R2.

Dpi / Tissue	1	3	5	7	10	14	21	28	Index
Duo	2	3	3	3	2	2	0	0	15
JeJ	1	3	3	3	1	0	2	0	13
Ileum	1	2	3	3	2	2	2	0	15
Caecum	1	3	3	3	3	0	3	3	19
Rectum	0	3	3	3	2	2	2	2	17

Total isolation indices 79

Table 3. Virus isolation indices of pooled (n=5) intestinal regions of normal chicks infected subcutaneously when day-old with reovirus strain R2.

Dpi / Tissue	1	3	5	7	10	14	21	Index
Duo	0	2	3	0	2	0	0	7
Jej	3	3	3	1	0	0	0	10
Ileum	2	2	3	2	2	0	0	11
Caecum	1	2	3	2	0	1	3	12
Rectum	3	3	3	3	3	1	0	16

Total isolation indices 56

Table 4. Virus isolation indices of pooled (n=5) intestinal regions of Bx chicks infected subcutaneously when day-old with reovirus strain R2.

Dpi / Tissue	1	3	5	7	10	14	21	28	Index
Duo	0	0	3	3	0	2	2	0	10
Jej	0	0	3	3	2	0	0	0	8
Ileum	0	0	3	3	2	2	0	0	10
Caecum	0	0	3	3	3	3	0	0	12
Rectum	0	0	2	3	3	3	0	0	11

Total isolation indices 51

5.3.3 Virus titration

A comparison of titres in the intestinal tissues of the normal and the Bx chicks infected with reovirus is given in Figures 2 to 11.

Virus titration results indicate that the normal chicks infected orally attained higher peak virus titres than Bx chicks in 4/5 intestinal tissues sampled (Figures 2 to 6). On the other hand, the Bx chicks had virus persisting for longer periods compared to the normal in 4/5 intestinal tissues.

Among the chicks infected subcutaneously (Figure 7 to 11), the normal and Bx groups had comparable peak virus titres. However, in a similar pattern to chicks infected orally, the subcutaneous ones also had virus persisting for longer periods in the Bx group.

Oral infection

Duodenum

Peak titre for normal birds was almost 3 log_{10} higher than Bx birds, but there was evidence of reappearance of virus at day 21 pi in the Bx group (Figure 2).

Jejunum

Peak titre for normal birds was some 2 log_{10} higher than the peak for Bx birds, but the latter was delayed by about one day (Figure 3).

Ileum

While peak titres were similar in normal or Bx groups, recovery for for the latter was again delayed (Figure 4).

Caecum

Highest titres in the Bx caeca were approximately 1.0 log higher than those in the normal group. Furthermore, the Bx viral persistence was prolonged for one week more (Figure 5).

Rectum

Again the titres from normal were higher than those from the Bx birds but a second burst of virus was detected in the Bx group on day 21 pi (Figure 6).

Subcutaneous infection

Duodenum

Virus recovery was delayed in Bx birds until day 5 pi. Peak titres in Bx and normal birds were similar but virus recovery was delayed by 2 days in the Bx group (Figure 7).

Jejunum

Virus recovery was delayed in Bx birds until day 5 pi, although peak titres were similar in Bx and normal birds. Virus persisted for one week with the Bx chicks with a second peak occurring on day 14 pi. (Figure 8).

Ileum

Virus recovery was delayed in Bx group until day 5 pi. Although peak titres were similar in Bx and normal birds, there was although lower, a second peak in the Bx chicks on day 14 pi. (Figure 9).

Caecum

Virus recovery was delayed in Bx group until day 5 pi. Peak titres in Bx and normal were similar but virus persisted for one extra week in the Bx group (Figure 10).

Rectum (Figure 11)

Virus recovery was delayed until day 5 pi although peak titres and persistence were similar in normal or Bx groups.

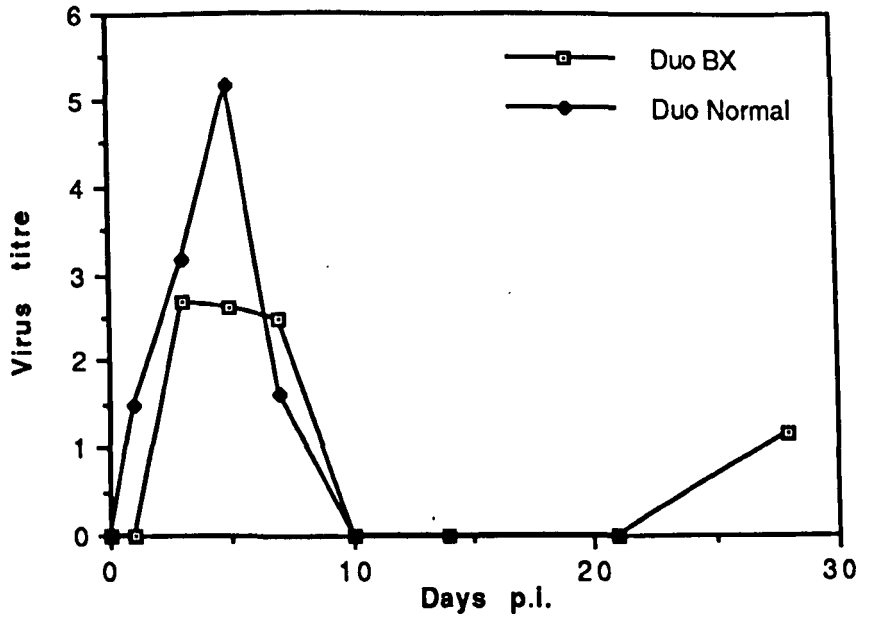

Figure 2. A comparison of the virus titres ($log_{10}TCID_{50}$ per 50µl) in the duodenum of Bx and normal chicks infected orally when day-old with reovirus strain R2.

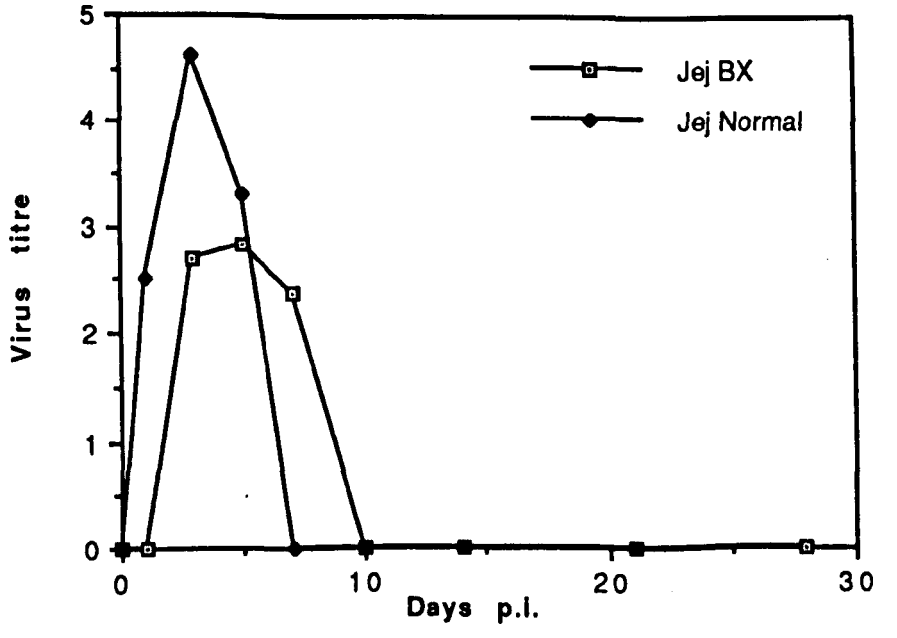

Figure 3. A comparison of the virus titres ($log_{10}TCID_{50}$ per 50µl) in the jejunum of Bx and normal chicks infected orally when day-old with reovirus strain R2.

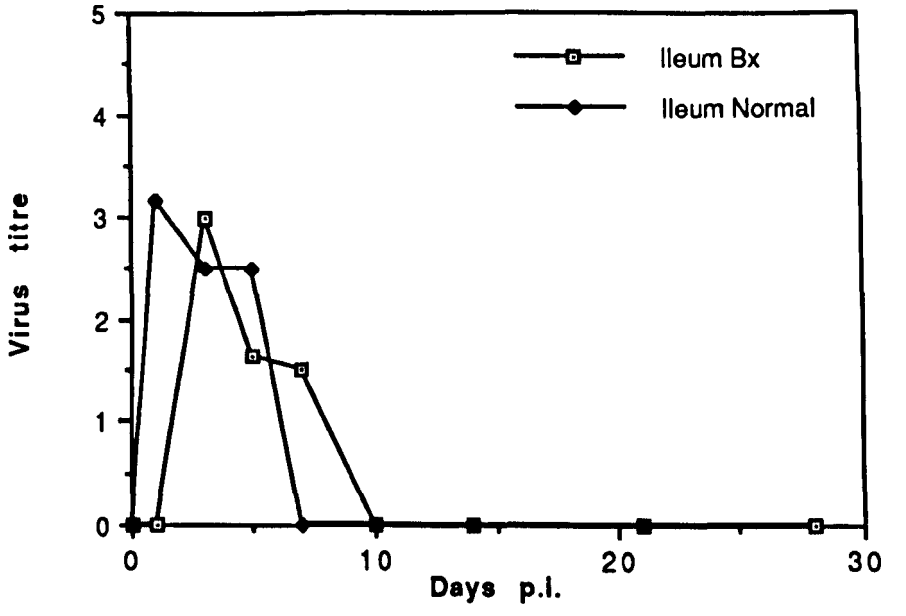

Figure 4. A comparison of the virus titres ($log_{10}TCID_{50}$ per 50µl) in the ileum of Bx and normal chicks infected orally when day-old with reovirus strain R2.

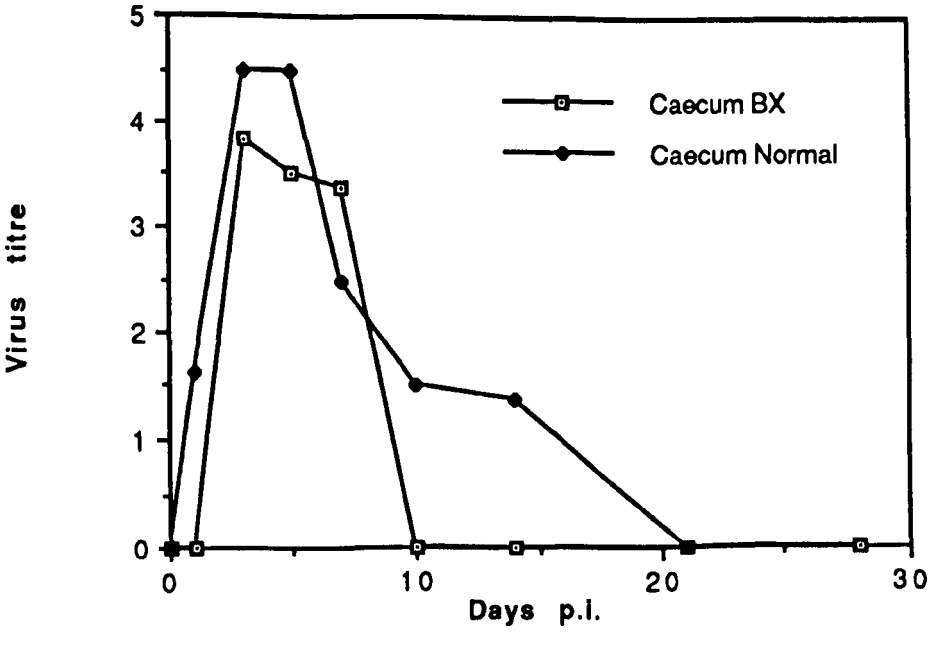

Figure 5. A comparison of the virus titres ($log_{10}TCID_{50}$ per 50µl) in the caecum of Bx and normal chicks infected orally when day-old with reovirus strain R2.

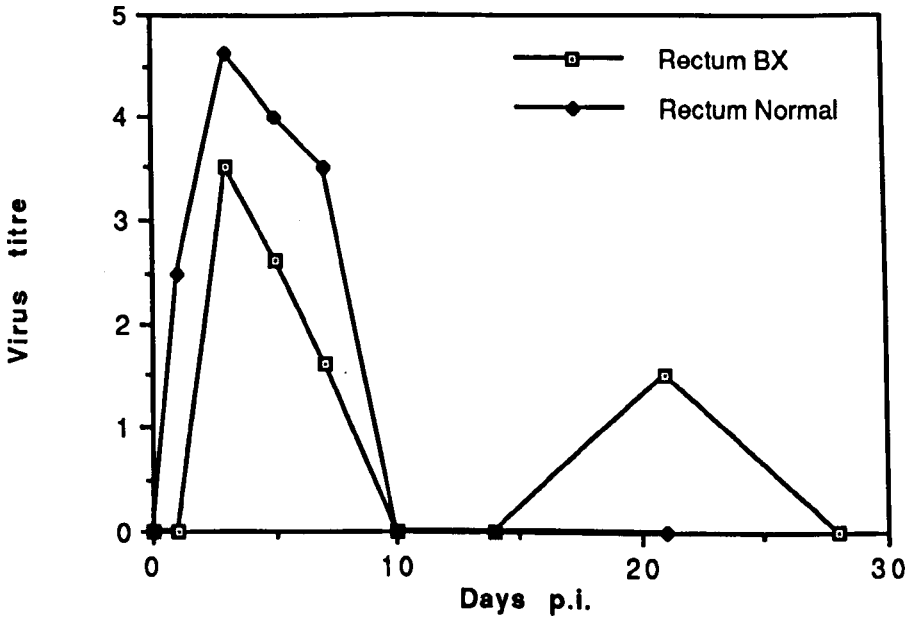

Figure 6. A comparison of the virus titres ($log_{10}TCID_{50}$ per 50µl) in the rectum of Bx and normal chicks infected orally when day-old with reovirus strain R2.

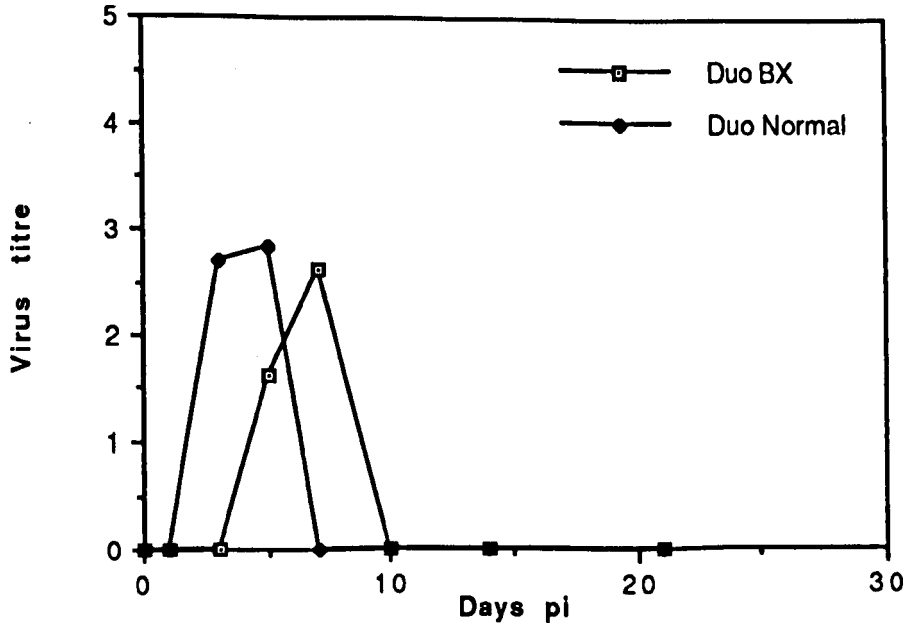

Figure 7. A comparison of the virus titres ($log_{10}TCID_{50}$ per 50µl) in the duodenum of Bx and normal chicks infected subcutaneously when day-old with reovirus strain R2.

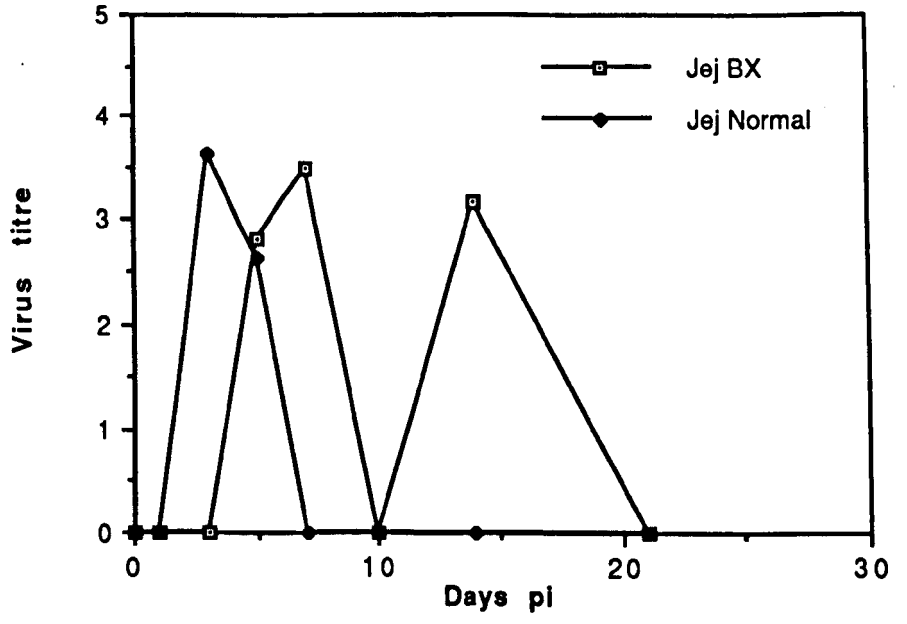

Figure 8. A comparison of the virus titres ($log_{10}TCID_{50}$ per 50µl) in the jejunum of Bx and normal chicks infected subcutaneously when day-old with reovirus strain R2.

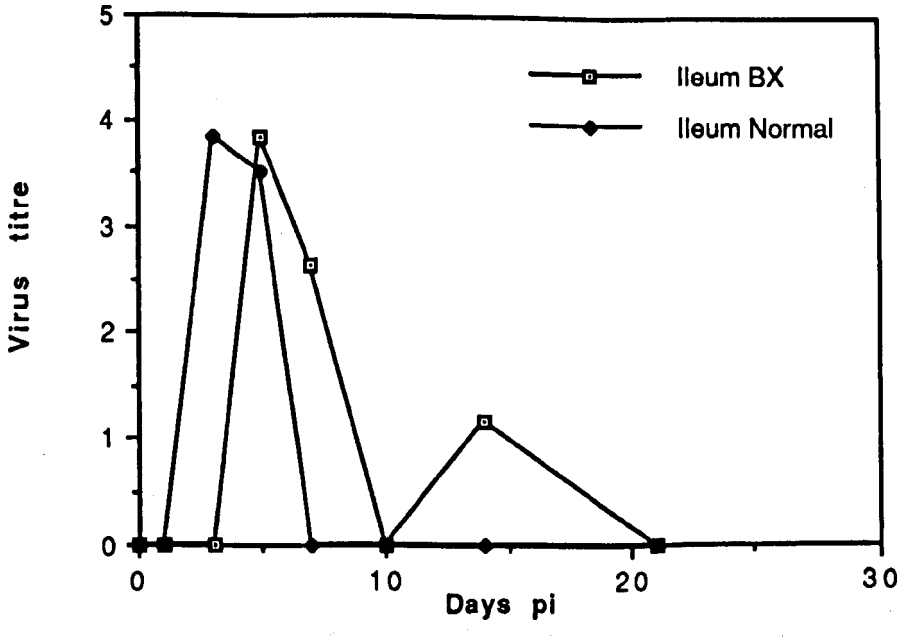

Figure 9. A comparison of the virus titres ($log_{10}TCID_{50}$ per 50µl) in the ileum of Bx and normal chicks infected subcutaneously when day-old with reovirus strain R2.

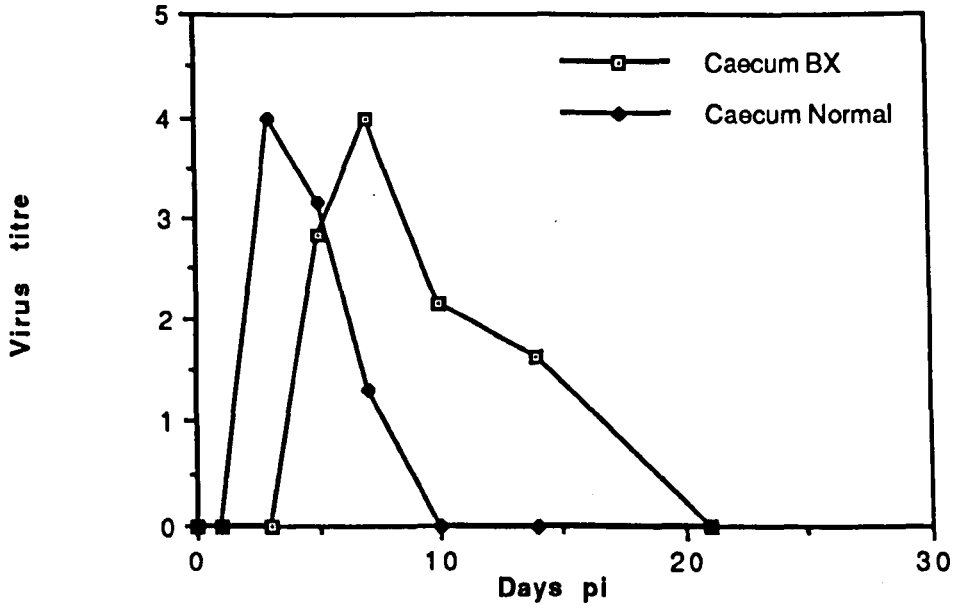

Figure 10. A comparison of the virus titres ($log_{10}TCID_{50}$ per 50µl) in the caecum of Bx and normal chicks infected subcutaneously when day-old with reovirus strain R2.

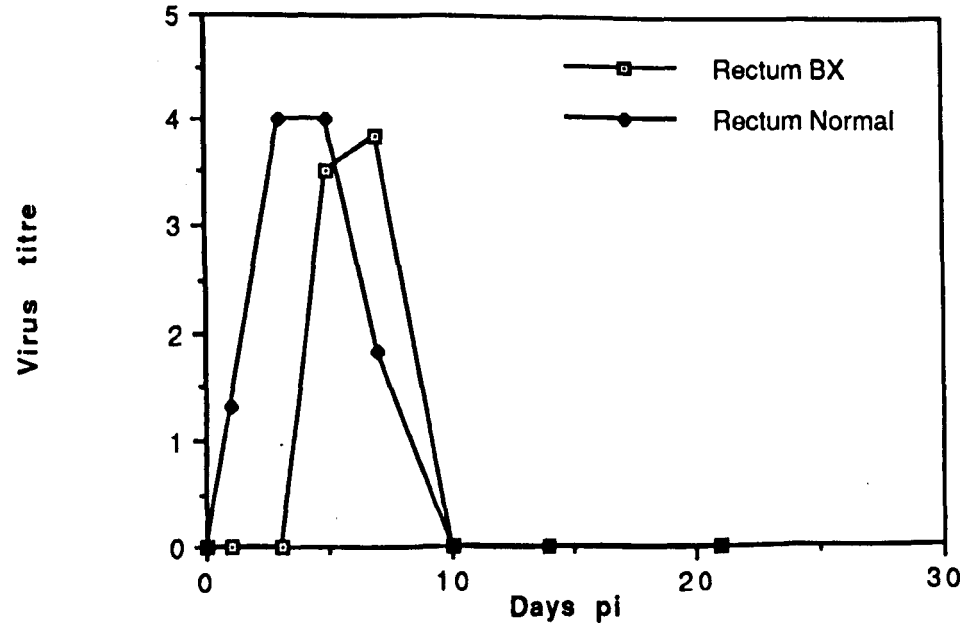

Figure 11. A comparison of the virus titres ($log_{10}TCID_{50}$ per 50µl) in the rectum of Bx and normal chicks infected subcutaneously when day-old with reovirus strain R2.

5.3.4 Immunoglobulin assays

5.3.4.1 Reovirus-specific IgA and IgG in intestinal contents

There was no detectable IgA or IgG in the intestinal contents of Bx chicks infected orally or subcutaneously with avian reovirus strain R2 (Figures 12 and 13 respectively). There were also no virus-specific IgA or IgG in intestinal contents of normal chicks infected orally or subcutaneously at day old (not shown).

5.3.4.2 Reovirus-specific IgG in sera

Comparisons of the reovirus-specific IgG in sera of normal or Bx chicks infected orally or subcutaneously when day-old with R2 are shown in Figures 14 and 15.

Production of reovirus-specific IgG in normal chicks infected orally showed the expected rise from 7 to 21 days pi. In the Bx group, there was no detectable IgG except for day 21 pi and even then it was about one third less than that in the normal chicks.

Among the subcutaneously infected chickens (Figure 15), the normal group showed a steady rise in virus-specific IgG from 7 to 21 days pi while the Bx group had no detectable virus-specific IgG until day 21 pi, when its value was approximately 1/5 that in the normal chicks.

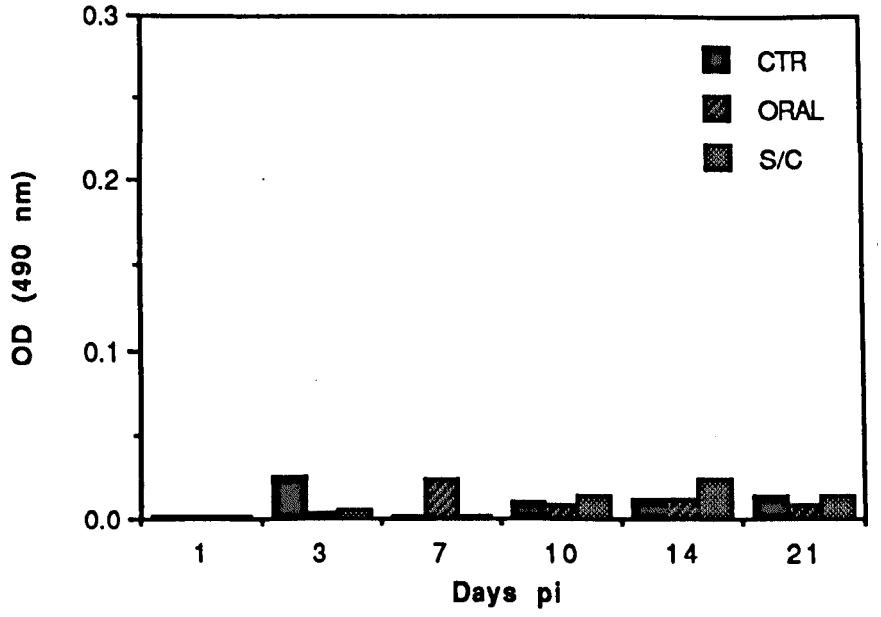

Figure 12. Reovirus-specific IgA in intestinal contents of Bx chicks infected orally or subcutaneously when day-old with strain R2.

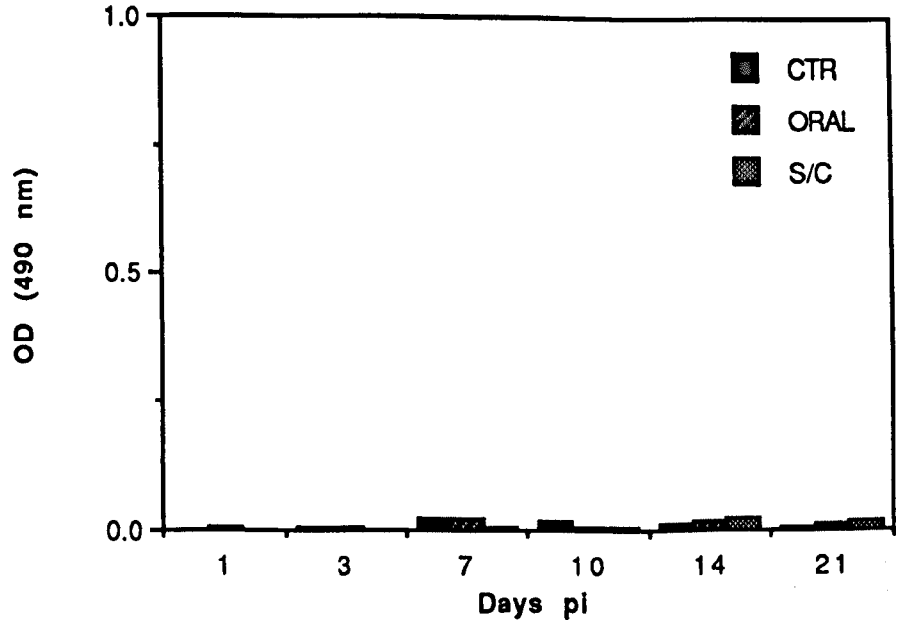

Figure 13. Reovirus-specific IgG in intestinal contents of Bx chicks infected orally or subcutaneously when day-old with strain R2.

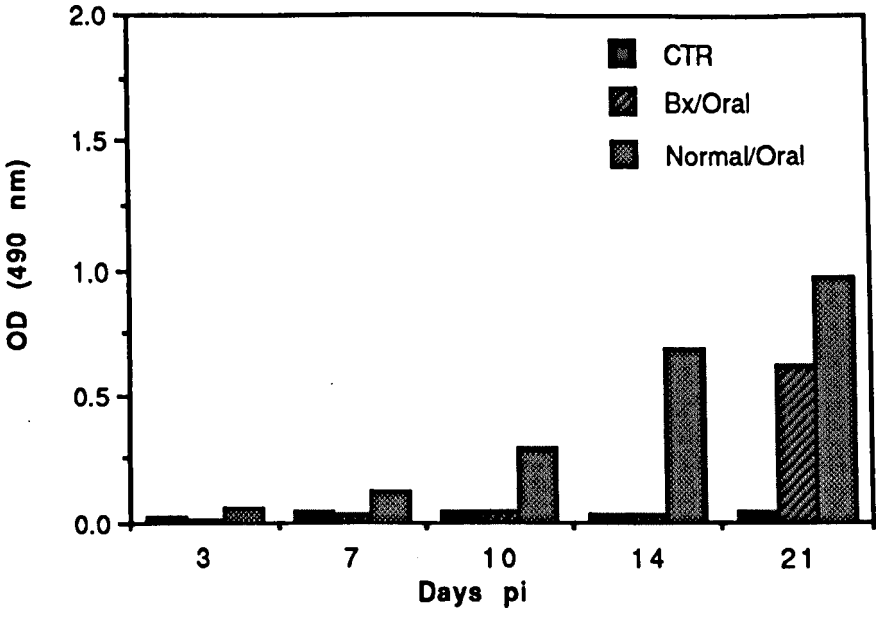

Figure 14. A comparison of reovirus-specific IgG in sera of Bx or normal chicks infected orally at DO with reovirus strain R2.

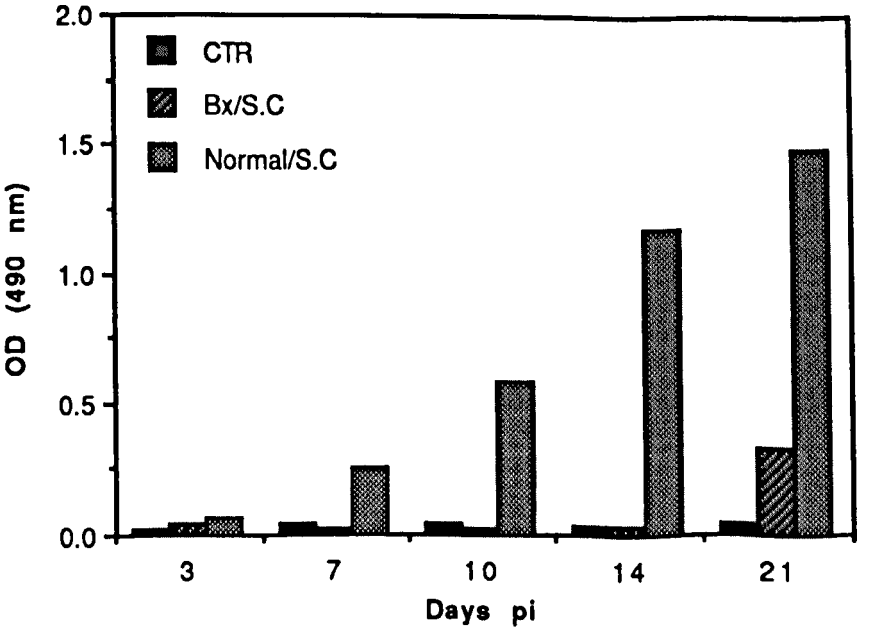

Figure 15. A comparison of reovirus-specific IgG in sera of normal or Bx chicks infected subcutaneously when day-old with avian reovirus strain R2.

EXPERIMENT 2. EFFECT OF B-CELL IMMUNOSUPPRESSION ON INTESTINAL AND SYSTEMIC IMMUNE RESPONSES OF CHICKS INFECTED WHEN ONE WEEK-OLD WITH AVIAN REOVIRUS STRAIN R2

In Experiment 1 of this chapter, no reovirus-specific IgA was detectable in the intestinal contents of chicks infected when day-old. This finding confirmed the fact that day-old chicks are less immunocompetent than 3 week-old chicks (see Chapter 4). In order to better examine the effects of immunosuppression in the gut, older chicks with the ability to produce intestinal IgA were chosen, and hence, this experiment, in which one-week-old chicks were used.

5.4 Materials and methods

5.4.1 Chicks

One week-old SPF chicks were used.

5.4.2 Virus

Avian reovirus strain R2 was used.

5.4.3 Bursectomy

Although hormonal Bx was effective as shown by the brucella agglutination test (Experiment 1), there was incomplete agammaglobulinaemia in sera. So, in Experiment 2 a combination of hormonal and chemical bursectomy was used.

Hormonal bursectomy was applied *in ovo* as in Experiment 1 together with chemical bursectomy as described by Kibenge et al. (1987) as follows. On each of the first three days after hatch, chicks which had been hormonally treated were injected

intraperitoneally (i.p) with 4 mg of cyclophosphamide monohydrate (Sigma) in 0.5 ml of distilled water.

5.4.4 Experimental design

Thirty two one-week old SPF Bx chicks were divided into two groups of 16 birds each. One group was infected orally with 0.3 ml of medium M199 containing 3.18 $\log_{10}$ $TCID_{50}$ per 50µl of avian reovirus strain R2 while a second was infected with the same dose of virus subcutaneously. Another batch of 32 SPF normal chickens were divided into two equal groups each of which were infected as previously stated, to represent the oral and subcutaneous groups of non-bursectomised birds.

On days 3, 7, 14 and 21 and pi, four birds were taken from each group and bled, after which they were humanely killed. The birds were autopsied and the intestinal tracts removed. In this experiment representative pieces of the duodenum, jejunum, ileum and caecum were collected from each bird and pooled to give a single gut sample for virus isolation and titration per bird. From each of the intestinal tract pieces a sample of the intestinal contents was gently squeezed out, pooled and kept at -70^0C until immunoglobulin assays for IgA were done.

5.4.5 Intestinal tract sampling and intestinal content treatment

These were performed as stated in Chapter 4.

5.4.6 Virus isolation and titration

These were performed as already described in Chapter 4. except that the intestinal tissue samples were not pooled. Results were analysed by the student t test using Starview, a statistical package for MacIntosh computers.

5.5 Results

There was no mortality in the cyclophosphamide treated chicks.

5.5.1 Virus isolation and titration

Figures 16 and 17 show the virus titres in the intestinal tissues of normal and Bx chicks infected orally or subcutaneously when one week-old with avian reovirus strain R2.

In the orally infected groups (Figure 16), virus was isolated from the normal birds only on 3 dpi while in the Bx ones, virus was recovered on 3 and 7 days pi. Peak titres were identical among the orally infected groups. In the subcutaneously infected groups (Figure 17), the Bx birds showed a delay in virus isolation and titratable virus was detected on 7 day pi only. However, the Bx chicks had higher peak virus titres than the normal chicks.

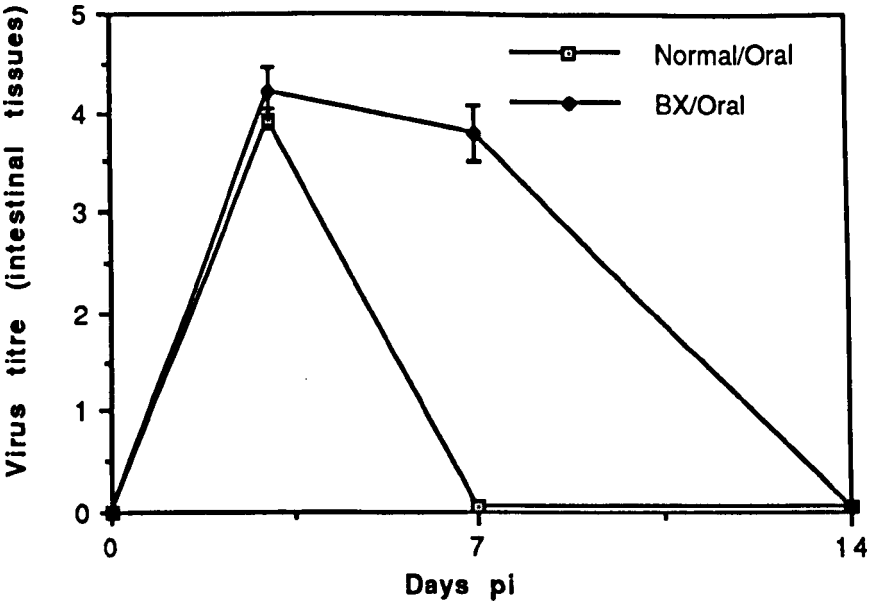

Figure 16. A comparison of virus titres from the intestinal tissues of normal and Bx chicks infected orally when one week-old with avian reovirus strain R2.

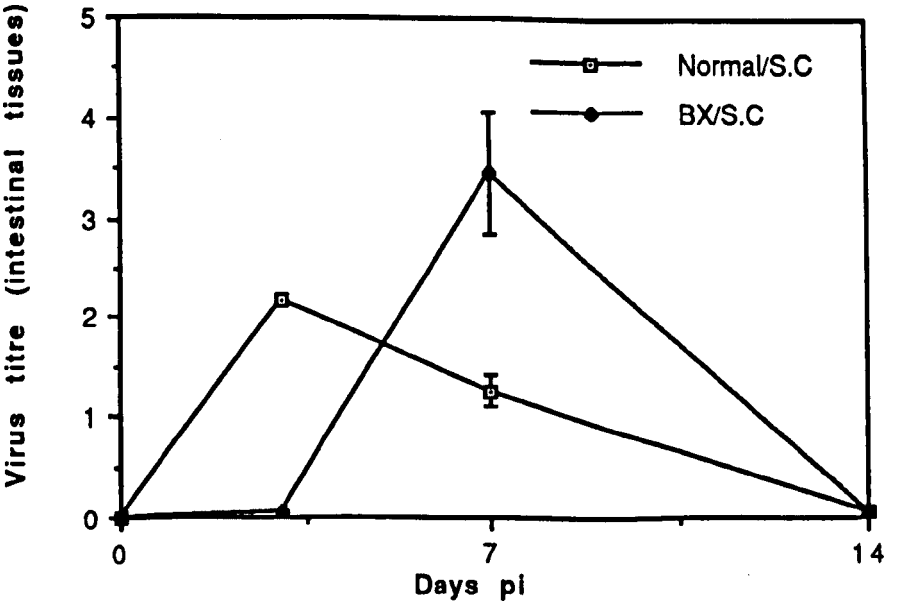

Figure 17. A comparison of virus titres from the intestinal tissues of normal and Bx chicks infected subcutaneously when one week-old with avian reovirus strain R2.

5.5.2 Immunoglobulin Assays

5.5.2.1 Reovirus-specific IgA

Comparisons of IgA OD values in the intestinal contents of the normal and Bx chicks infected orally or subcutaneously at one-week of age with avian reovirus strain R2 are shown in Figures 18 and 19.

Reovirus-specific IgA was not detected in Bx chicks infected orally, while in the normal chicks, IgA was detectable in increasing order from days 14 to 21 pi (Figure 18). Among the subcutaneously infected groups, there was no detectable IgA in either the Bx or normal chickens (Figure 19).

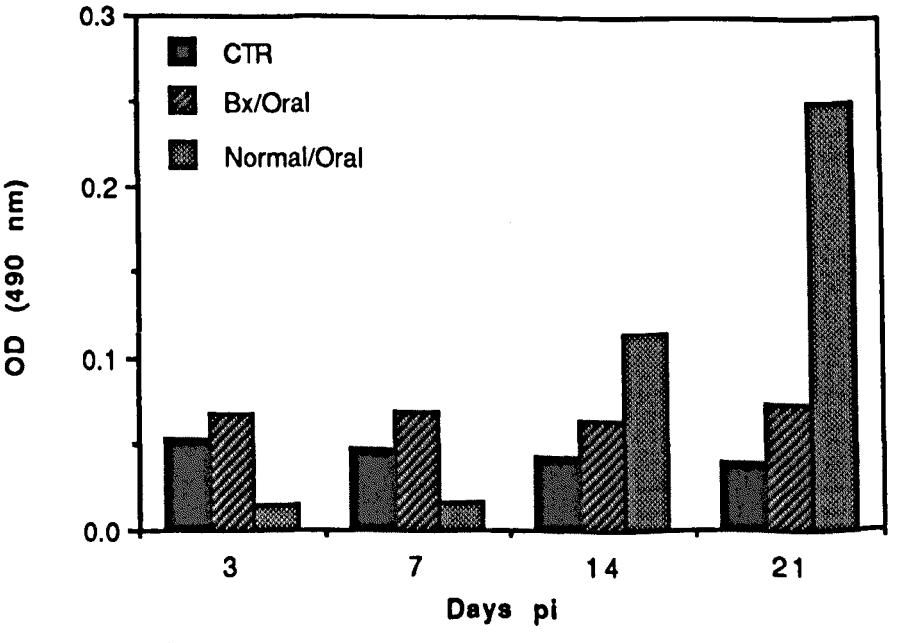

Figure 18. A comparison of reovirus-specific IgA in the intestinal contents of normal and Bx chicks infected orally when one week-old with reovirus strain R2.

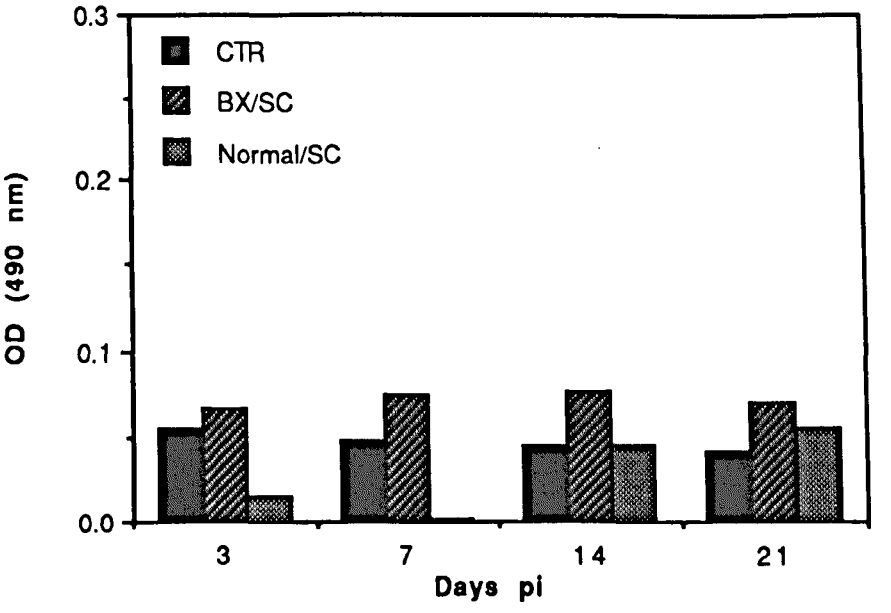

Figure 19. A comparison of reovirus-specific IgA in the intestinal contents of normal and Bx chicks infected subcutaneously when one week-old with reovirus strain R2.

5.5.2.2 Reovirus-specific IgG

Comparisons of the IgG OD values in sera of the normal and Bx birds infected orally or subcutaneously at one-week age with avian reovirus strain R2 are shown in Figures 20 and 21.

As expected, reovirus-specific IgG in sera of normal chicks infected orally rose from days 7 to 21 pi, while IgG in the Bx chicks was detected only on day 21 pi and at a much lower OD value than that of the normal birds (Figure 20).

In the subcutaneously infected groups virus-specific IgG of the Bx chicks showed a similar pattern to that of chicks infected orally (Figure 21). Thus, the rise in OD value of normal chicks infected subcutaneously when one-week old started on day 7 pi and continued up to the end of the experiment, while the OD values of the Bx group were detectable on day 21 pi only and at less than half that of the normal group.

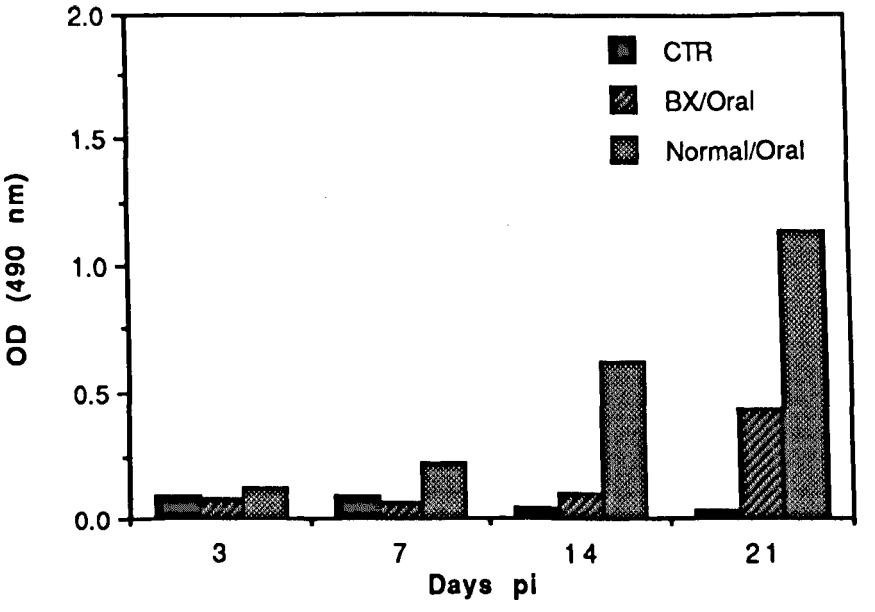

Figure 20. A comparison of reovirus-specific IgG in the sera of normal and Bx chicks infected orally at one week-old with reovirus strain R2.

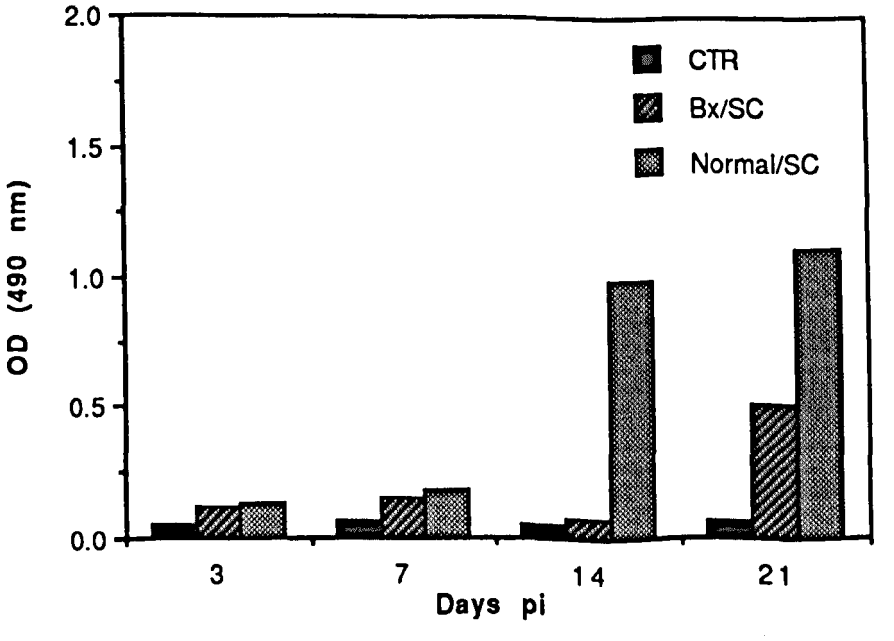

Figure 21. A comparison of reovirus-specific IgG in the sera of the normal and Bx chicks infected subcutaneously at one week-old with reovirus strain R2.

5.6 Discussion

The results presented here indicate that hormonal bursectomy with testosterone as used in experiment one and a combination of testosterone and cyclophosphamide in the second experiment did cause B-cell immunossuppresive effects. The use of hormonal bursectomy alone has been documented by a number of authors (Glick and Sadler, 1961; May and Glick, (1964); Hirota et al., (1976); Okoye and Uzoukwu (1990), while the combined use of hormonal and chemical bursectomy has also been attempted (Lillehoj, 1987; Yuasa et al., 1988).

The tissue isolation scores of orally infected day-old chicks did not show any major difference between the normal and Bx groups. However, in the subcutaneously infected day-old chicks, there was a delay in virus replication in the tissues of chicks after infection when day-old or one week of age of about four days in the Bx group. The reason for this are not known but it is worth speculating on the possible mechanisms.

Virus inoculated by the subcutaneous route has to travel via the systemic blood and/or lymphatic route to reach the intestinal tract and it is possible that some of the components which enhance the initial replication within the transport system are temporarily affected by immunosuppression. The hormonal and chemical effects on physiological processes other than the B-cell suppression can not be ruled out.

Virus titration results from the orally infected day-old chicks had higher peak virus titres among the normal group compared to those Bx. This may indicate that although reovirus was present in the intestinal tract of Bx chicks, it could not replicate to its full potential. Some of the cells which support its replication in the gut could have been affected by treatment. Despite these reduced peak virus titres, there was persistance of virus in the duodenum, jejunum, ileum and rectum of the Bx chicks and also from the duodenum, jejunum, ileum, caecum of those infected

subcutaneously. This persistance of virus confirms earlier findings by Kibenge et al. (1987) and Moradin et al. (1990) who isolated reovirus for a longer time in immunocompromised chickens.

In chicks infected orally or subcutaneously when day-old, the Bx groups showed a delayed production of reovirus-specific IgG, appearing on 21 days pi only, and even then in reduced amount compared to the normal chicks. These results were also similar to those obtained by Springer et al. (1983), Kibenge et al. (1987) and Moradin et al. (1990) and may indicate that this B-cell suppression is transient.

As there was no local immune response detected in the intestinal contents of normal or Bx chicks infected orally or subcutaneously when day-old, a similar experiment was set up using slightly older chicks which would circumnavigate the problem of poor local immune response in day old chicks (see Chapter 4). In order to avoid the possibility that perhaps hormonal bursectomy was not fully effective, a combined hormonal and chemical bursectomy was attempted.

The overall results of virus isolation and titration in Experiment 1 and those in Experiment 2 are not different, which indicates that both methods of immunosuppression had similar effects on the humoral immune system. In the Bx chicks infected when one-week old, there were higher virus titres at day 7 pi compared to normal chicks. Although age-related resistance could have had an effect, these differences in intestinal virus titres may have also been due to reduced resistance due to immunosuppression.

The results in Experiment 2 show that there was no detectable reovirus-specific IgA in Bx chicks infected orally when one-week old, while there were substantial amounts of IgA in the normal chicks infected orally. The effect of immunosuppression clearly extended to the local immune responses of the gut and presumably was responsible for the greater viral replication in the one week old birds.

There was no virus-specific IgA in the intestinal contents of either the normal or Bx chickens after infection subcutaneously when one week old. This lack of IgA in one week-old infected birds when inoculated by the subcutaneous route is likely to be associated with the relatively younger age of chickens used, because in our earlier work (Chapter 4) intestinal virus-specific IgA was detected in chicks infected subcutaneously when 3 week-old but not in one week-old by the same route. Bursectomy, whether chemical or surgical or combined in chickens has been reported to result in a decrease or delayed total serum IgA levels (Martin and Leslie, 1973; Perry and Bienenstock, 1973; Schlink et al., 1992).

There was a similarity in the pattern of reovirus-specific IgG of Bx chicks infected orally or subcutaneously. Likewise there was a similarity among the normal chicks infected orally or subcutaneously. Thus immunosuppression substantially interfered with virus-specific IgG production irrespective of the route used. However the interference was not complete, since by day 21 pi some IgG was detected although at lower level. It has reported that hormonal and cyclophosphamide treatment may not completely make chicks agammaglobulinemia (Lillehoj, 1987).

Under field conditions many agents such viruses (IBD), toxins, and dietary vitamin deficiencies may cause immunosuppression and with a constant challenge of field reoviruses, tenosynovitis could easily occur even after vaccination.

This study clearly shows that antibody-mediated mechanisms play a significant role not only in the pathogenesis but also in intestinal and systemic immune responses in chicks infected with reovirus.

CHAPTER 6

THE ROLE OF THE HARDERIAN GLAND IN REOVIRUS IMMUNE RESPONSE IN CHICKEN AND THE USE OF THE OCULAR ROUTE TO ESTABLISH RESISTANCE TO RE-INFECTION TO AVIAN REOVIRUS

CONTENTS PAGE

CHAPTER 6

THE ROLE OF THE HARDERIAN GLAND IN REOVIRUS IMMUNE RESPONSE IN CHICKEN AND THE USE OF THE OCULAR ROUTE TO ESTABLISH RESISTANCE TO RE-INFECTION TO AVIAN REOVIRUS

6.1 Introduction

The harderian gland (HG) of the chicken is a strap-like structure situated ventrally and posterio-medially to the eyeball and has a single duct which leads to the medial angle of the nictitating membrane (Wight et al., 1971). It is a major lymphoid tissue associated with antibody-producing immune responses in the chicken (Bang and Bang, 1968; Bienenstock, et al., 1973; Davelaar et al., 1982). The HG and other associated lymphoid organs (lacrimal gland and nasal gland) collectively known as the head-associated lymphoid tissues (HALT), constitute a local immune environment (Montgomery et al., 1991). Surgical removal of the HG has been shown to reduce the amount of lacrimal immunoglobulin and specific antibody titre (Survashe and Aitken, 1977; Davelaar and Kouwenhoven, 1980; Baba et al., 1988).

Furthermore, Baba et al. (1990) found that lacrimal IgA almost disappeared after surgical removal of the HG and immunoglobulins produced by the HG cells was detected in saliva but not in the trachea. Using explanted HG cultured *in-vitro*, immunoglobulin production was shown to consist mostly of IgA, while amounts of IgG and IgM were very low (Baba et al., 1990). These findings confirmed the fact that lacrimal IgA is produced locally in the HG while IgG and IgM are mainly transuded from blood into the tears.

The involvement of the HG in the local immune response of specific viral infections of the chicken has been reported for infectious bronchitis (IB) virus, (Davelaar et al., 1982; Russell and Koch, 1993) and in Newcastle disease (ND) virus, (Russell and Koch, 1993; Russell, 1993).

Recently, coarse-spray (CS) administration of a commercial reovirus vaccine (S1133) has been shown to induce effective protection against the 2408 and CO8 enteric isolates of avian reovirus (Giambrone et al., 1992). This method of vaccine administration is popular with large broiler producers, where it is used widely with ND and IB vaccines. Although the CS method is not identical to the intra-ocular route, in both methods, the vaccine given this way gets into contact with the HG primarily, before reaching other sites, for example the gut.

Various studies have described the distribution of reovirus in tissues of the chicken (Menendez et al., 1975; Jones et al., 1989; Jones et al., 1994; Mufarrej et al., 1996) but there has not been any mention of the HG.

The objectives of the present study were (a) to establish whether the HG can support reovirus replication and subsequently produce a local immune response and (b), to compare the local and systemic immune responses in chickens after infection by the eye-drop, oral or subcutaneous routes using a non-attenuated trypsin-resistant avian reovirus, strain R2.

EXPERIMENT 1. THE IMMUNE RESPONSE OF THE HARDERIAN GLAND AND ITS ASSOCIATED TISSUES AFTER INFECTION WITH AVIAN REOVIRUS

6.2 Materilas and methods

6.2.1 Chickens

One day-old SPF White leghorn chicks were used in all experiments. There were given water and food *ad libitum*.

6.2.2 Virus

Avian arthrotropic reovirus R2 which is trypsin-resistant has been described in Chapter 3.

6.2.3 Experimental design

One hundred and twenty five day-old SPF chicks were divided into five groups, one of 45 birds and four of 20 each (Table 1). The first group of 45 birds was inoculated by eye-drop (ED) at DO, each bird receiving 50µl of M199 medium containing 3.5 $\log_{10}$ $TCID_{50}$ per 50µl of reovirus. The inoculum was divided into 2 x 25µl volumes for each eye. Some of the chicks in this group were later challenged (see below) and hence referred to as the ED+Ch group. The second group of 20 was treated in a similar manner but were not challenged and are referred to as the ED group. The third group was not infected at DO but was later challenged (Ch), while the fourth group was inoculated orally at DO with 3.5 $\log_{10}$ $TCID_{50}$ per 50µl of virus, and these groups were referred to as the Ch and Or+Ch groups respectively. The fifth group of 20 birds was left uninfected throughout as controls (CTR).

The challenge dose of virus was 3.5 $\log_{10}$ $TCID_{50}$ per 50µl given orally on day 38 post-infection. In order to determine when initially

given virus could no longer be detected and before challenge, the Or+Ch group was swabbed via the cloacal on every other day until no more virus could be re-isolated.

Following the first infection, on days 3, 5, 10, 14, and 21, five birds from the first group (ED) were bled for serum and then their lacrimal fluid (tears) collected.

Representative samples of the gut (duodenum, ileum, and caecum) were collected and pooled for virus isolation and titration.

The remaining 20 birds were kept until they were challenged orally 38 days later, by which time virus from the original infection could no longer be isolated.

Following challenge, on days 3, 7, and 14, five birds from each group were humanely killed and sampled. Pieces of duodenum, ileum, caecum and rectum from each bird were pooled for virus isolation and titration. Intestinal contents were squeezed out of these gut pieces, pooled and treated for immunoglobulin assay as already described in Chapter 4.

6.2.4 Collection of tears and HG

This was facilitated by placing a few grains of sodium chloride on the eye to stimulate lacrimation and the tears were collected using an Eppendorf pipette. The birds were then humanely killed and the HG removed aseptically from both sides of the head and kept for virus isolation. Each HG was removed by making one cut from the medial canthus to the nostril and a second cut from the medial canthus to the angle of the mouth. The nictitating membrane and the adjacent skin were pulled backwards to expose the HG.

6.2.5 Virus isolation and titration

These were carried out as already described in Chapter 3.

6.2.6 ELISA for immunoglobulin assay

This was performed as already described in Chapter 3.

6.2.7 Virus-neutralisation test

This was done as already described in Chapter 4 part one.

Table 1. Outline of Experiment 1.

Group	Infection at DO	Challenge at D38
ED + Ch	ED	Oral
ED	ED	None
Ch	None	Oral
Or + Ch	Oral	Oral
CTR (control)	None	None

6.3 Results

6.3.1 A. Initial infection (immunisation)

6.3.1.1 Virus isolation and titration

Virus isolation from cloacal swabs of chicks infected orally when day old (Group Or+Ch) ceased on day 31 pi (Table 2).

Virus was present in the HG tissues sampled on days 3, 5, 7 and 10 pi. and in the intestinal tract on days 3, 5, and 7 pi only. All tissues from non-infected birds were negative. Virus titration results from the HG and the pooled intestinal tissues are shown in Table 3.

Isolation of virus from the Harderian gland in low but consistent titres from day 3 to 7 pi indicated that there was a low level of replication in this organ. In contrast, virus titres in the intestinal tract were in decline from days 3 to 5 pi.

Table 2. Virus shedding via cloacal swabs of 20 chicks inoculated orally at day-old with avian reovirus strain R2.

		Days post-infection															
Passage level		3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33-38
Positive	1	17	19	15	17	13	17	11	11	5	4	3	0	0	0	0	0
	2	3	1	4	3	7	2	7	8	8	2	1	0	1	2	2	0
	3	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
Negative		0	0	1	0	0	1	2	1	7	13	16	20	18	18	18	20

I

Table 3. Virus titres (log_{10} $TCID_{50}$ per 50µl) in pooled Harderian gland and intestinal tissues of chickens infected by eye-drop when day-old (n=5).

	Days post-infection				
Tissue	3	5	7	10	14-21
Harderian gland	1.17	1.38	1.32	+	-
Intestinal tissues	3.32	2.63	+	-	-

+: positive but low titre; -: negative

6.3.1.2 Immunoglobulin assays

The appearance of reovirus-specific immunoglobulins IgA in tears, IgA in sera, IgG in tears and IgG in sera are shown in Figures 1, 2, 3 and 4 respectively.

Virus-specific IgA was detected in the tears from day 7 p.i. and the level sharply increased between days 14 and 21 pi (Figure 1). In sera, the presence of IgA was short-lived but it was detected between 7 and 14 days pi (Figure 2).

The sera and tears displayed similar profiles for IgG (Figures 3 and 4), although the quantities in sera and tears are not directly comparable because of differences in dilutions of test samples.

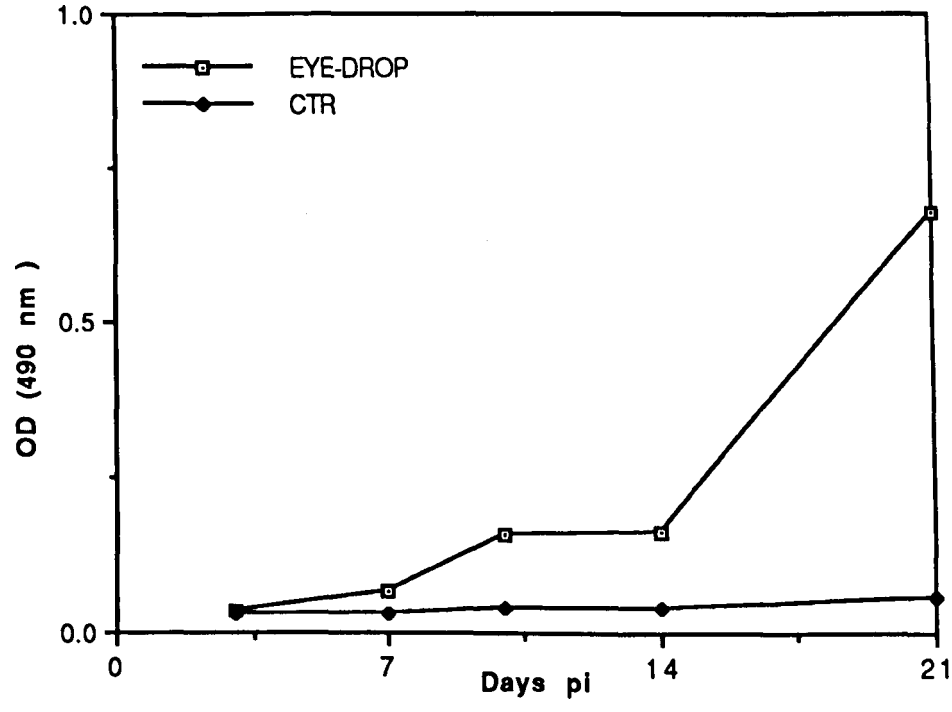

Figure 1. Reovirus-specific IgA in tears of chickens after eyedrop infection at day-old with reovirus.

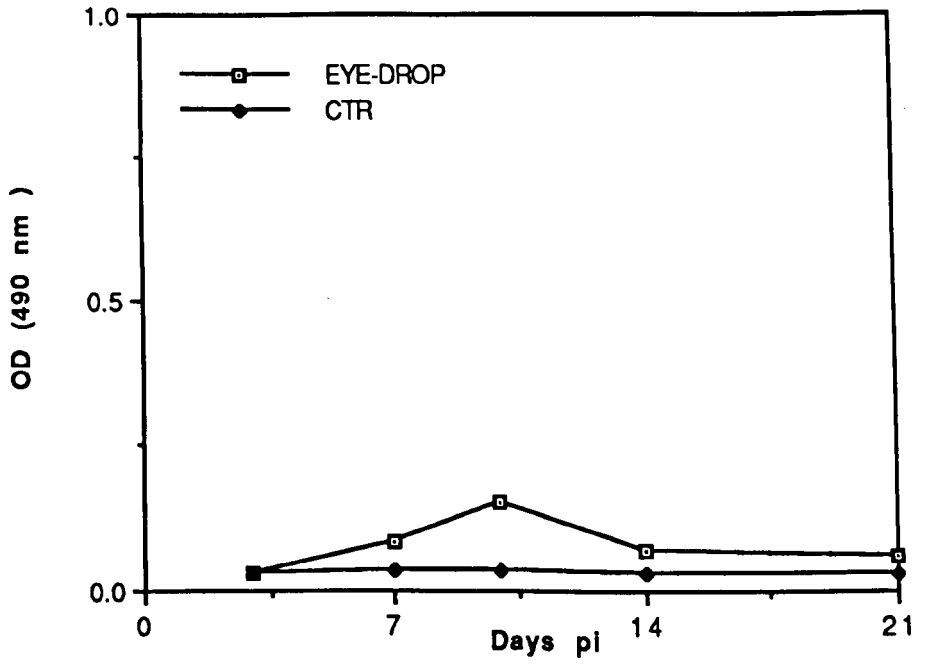

Figure 2. Reovirus-specific IgA in sera of chickens after eyedrop infection at day-old with reovirus.

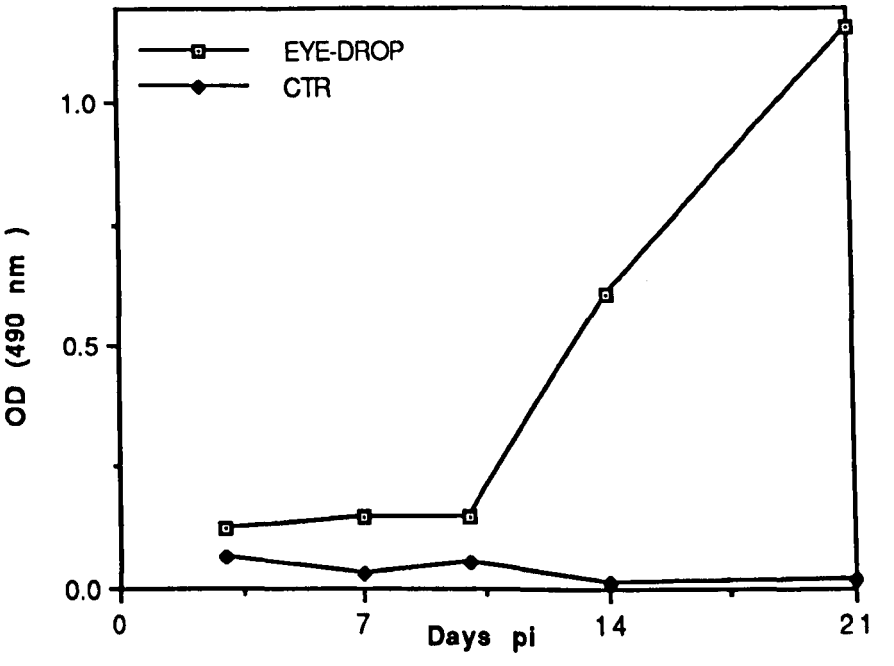

Figure 3. Reovirus-specific IgG in tears of chickens after eyedrop infection at day-old with reovirus.

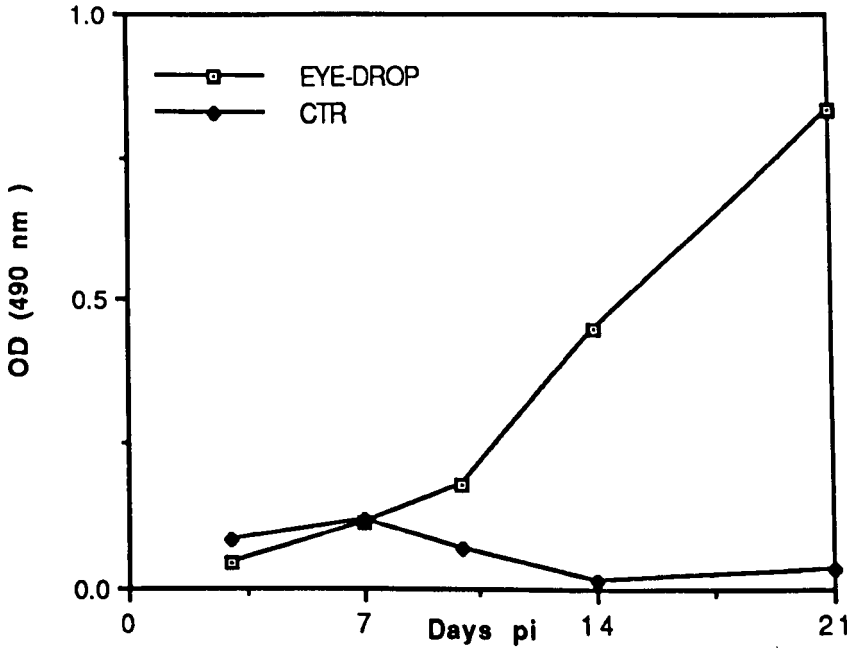

Figure 4. Reovirus-specific IgG in sera of chickens after eyedrop infection at day-old with reovirus.

6.3.1.3 Virus neutralising antibodies

The Neutralising antibody titre in pooled tears reached 5.0 log_2 by day 7 pi while that in sera was not detectable at this time (Figure 4). On day 14 pi, the titre in tears reached a maximum of 7.0 log_2, while that in sera had just reached 3 log_2. On day 21, pi the titre in the tears maintained the 7.0 log_2 mark while that in sera rose to reach 5.0 log_2.

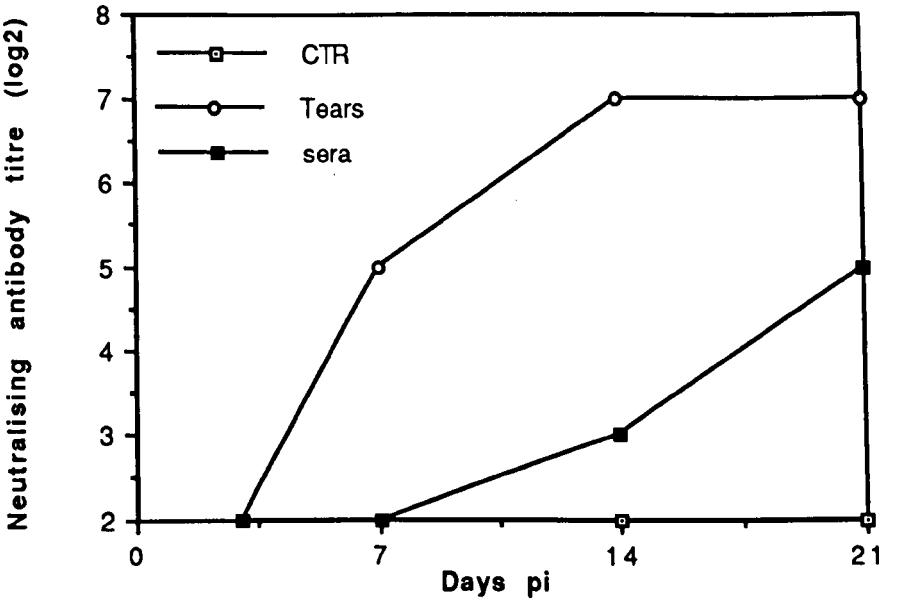

Figure 4. Virus neutralising antibodies (log_2) in pooled tears and sera from chicks infected by eye-drop or orally at day old with strain R2.

6.3.2 B. Post-challenge (pc) at 38 days pi

6.3.2.1 Virus isolation and titration

Virus isolation and titration results from the intestinal tissues post-challenge are shown in Table 4.

Virus was not recovered from any of the chicks which were initially infected at DO by either of the routes (ED, or Or), even after they had been challenged (ED+Ch, Or+Ch). In the group infected for the first time on day 38 (Ch), virus was recovered from all tissues of the intestinal tract, but only on day 3 post-challenge (pc).

Table 4. Virus isolation and virus titres (log_{10} $TCID_{50}$ per 50µl) in the intestinal tissues post challenge (pc).
[a]: - no virus isolated.

		Days post-challenge	
Group	Tissue	3	7-14
ED+Ch	Duodenum	-[a]	-
	Jejunum	-	-
	Ileum	-	-
	Caecum	-	-
	Rectum	-	-
ED	Jejunum	-	-
	Ileum	-	-
	Caecum	-	-
	Rectum	-	-
Ch	Duodenum	3.32	-
	Jejunum	3.53	-
	Ileum	3.20	-
	Caecum	3.32	-
	Rectum	2.87	-
Or+Ch	Duodenum	-	-
	Jejunum	-	-
	Ileum	-	-
	Caecum	-	-
	Rectum	-	-
CTR	Duodenum	-	-
	Jejunum	-	-
	Ileum	-	-
	Caecum	-	-
	Rectum	-	-

6.3.2.2 Immunoglobulin assays

The apprearance of reovirus-specific immunoglobulin IgA in intestinal contents, IgA in serum, IgG in intestinal contents and IgG in serum are shown in Figures 5, 6, 7 and 8 respectively.

6.3.2.2.1 IgA in intestinal contents

In the group infected for the first time on day 38 (Ch), a very low level of virus-specific IgA in the intestinal contents was detected on day 14 pc (Figure 5). No virus-specific IgA was detected in intestinal contents of chicks infected orally and challenged (Or+Ch) and those inoculated by the eye-drop (ED) but without challenge. Similarly, no virus-specific IgA was detected in the intestinal contents of the group initially infected at day-old by the ocular route and challenged at day 38 (ED+Ch).

6.3.2.2.2 IgA in sera

There was no detectable virus-specific IgA in sera of chicks infected by any of the routes and whether challenged or not (Figure 6).

6.3.2.2.3 IgG in intestinal contents

No virus-specific IgG was detected in the intestinal contents of any chicks tested with whichever treatment (Figure 7).

6.3.2.2.4 IgG in sera

Reovirus-specific IgG was detected in the sera of all the chicks which were infected by either of the routes, with or without challenge (Figure 8). The IgG detected on day 3 pc from previously infected groups (Or+Ch, ED and ED+Ch) was presumably residual, deriving from the first infection.

Virus-specific IgG was detected in the Ch group from days 7 to 14 pc. In the Or+Ch group the levels of IgG detected were similar on days 3 and 7 pc but they doubled in amount on day 14 pc.

The ED and ED+Ch groups had comparable levels of virus-specific IgG in sera on days 3 and 7 pc, but the latter also rose to twice as high on day 14 pc. Overall, the Or+Ch and ED+Ch groups had similar patterns of virus-specific IgG in sera.

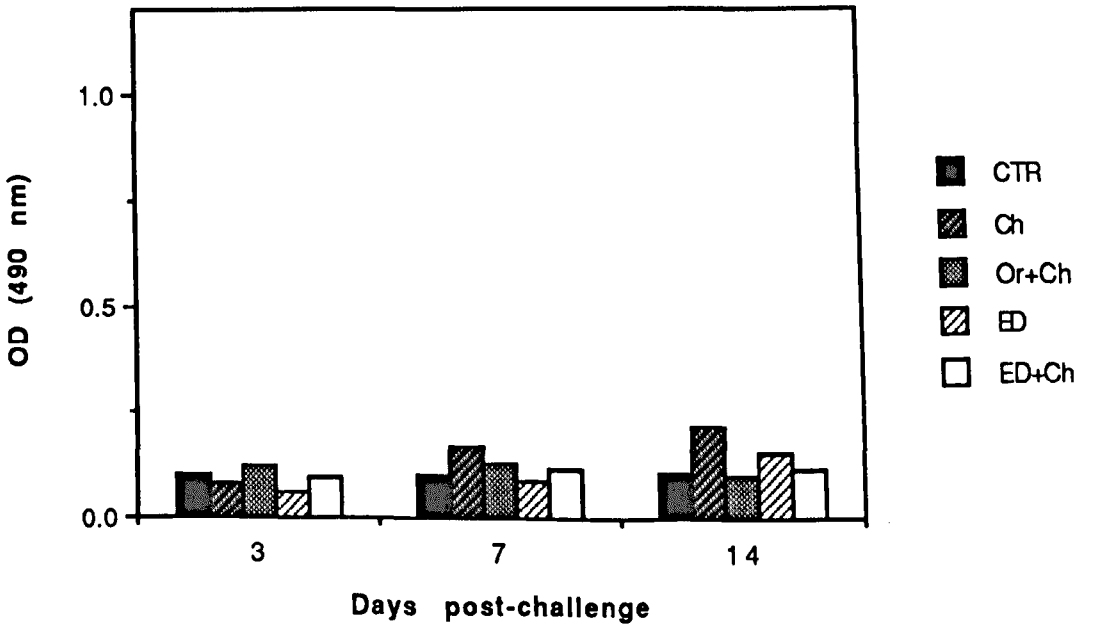

Figure 5. Reovirus-specific IgA in the intestinal contents of chicks infected orally or ocularly when day old and challenged at 38 days.

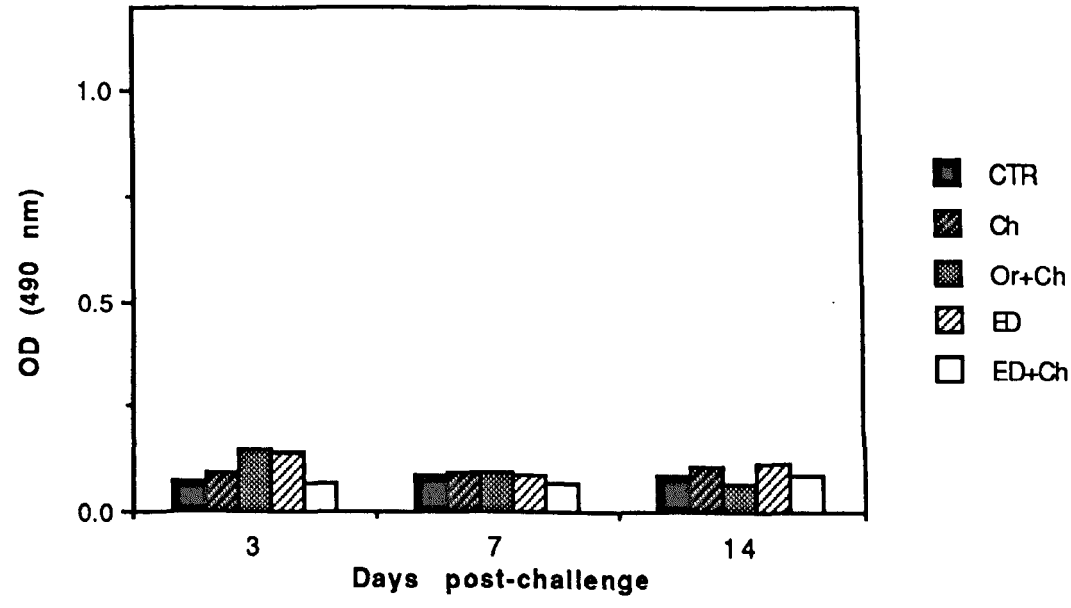

Figure 6. Reovirus-specific IgA in sera of chicks infected orally or by eyedrop when day-old and challenged after 38 days.

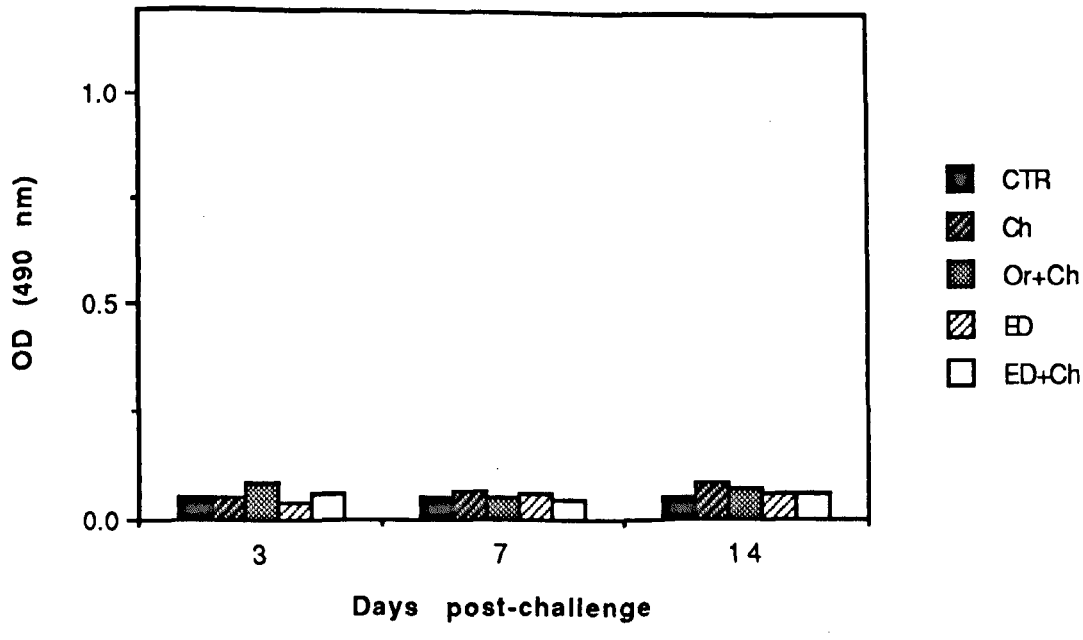

Figure 7. Reovirus-specific IgG in intestinal contents of chicks infected orally or ocularly when day-old and challenged at day 38.

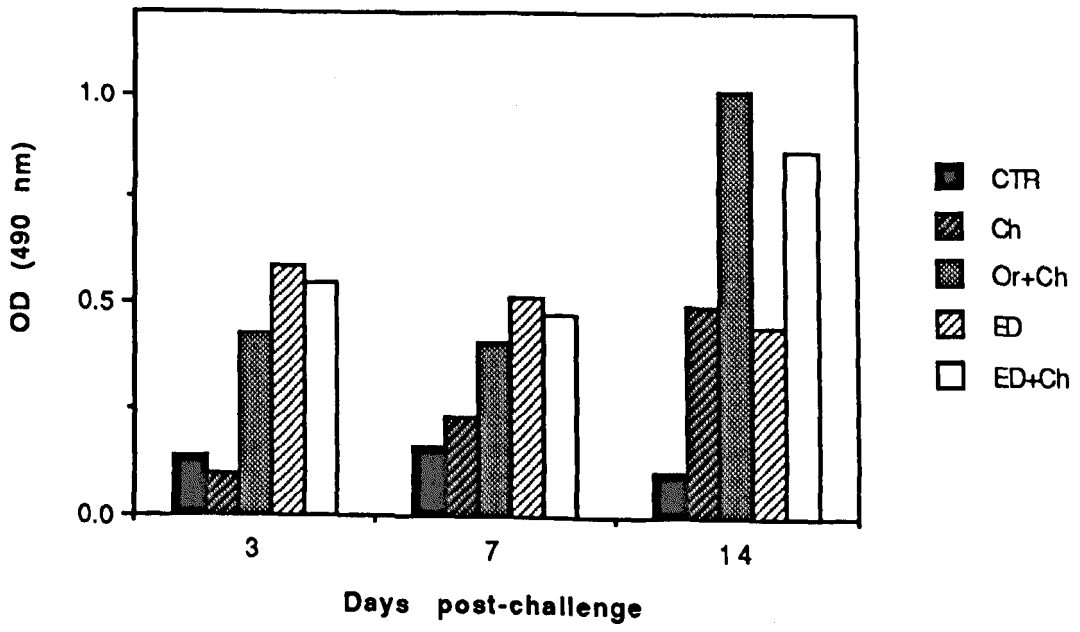

Figure 8. Reovirus-specific IgG in sera from chicks infected orally or ocularly when day-old and challenged at 38 days.

EXPERIMENT 2. A COMPARISON OF THE LOCAL AND SYSTEMIC IMMUNE RESPONSES IN CHICKENS TO AVIAN REOVIRUS AFTER INFECTION BY THE EYE-DROP, ORAL OR SUBCUTANEOUS ROUTES

The oral and the subcutaneous routes have been used for avian reovirus vaccinations for several years. The former has the difficulty of ensuring the correct dose each bird receives while the latter, is labour intensive since individual birds have to be injected. Recently coarse-spray application has been used in reovirus vaccination with some success (Giambrone et al., 1992). In Experiment 1 of this chapter, it has been shown that adminstration of virus by the eye-drop route can induce both local and systemic immune responses immediately after application which may protect against later challenge. The purpose of Experiment 2 is to compare the eye-drop route to the two conventional routes of adminstration.

6.4 Materials and methods

6.4.1 Chickens

One day-old SPF White leghorn chicks were used as described in Experiment 1.

6.4.2 Virus

Avian reovirus strain R2, already described in Experiment 1 was used.

6.4.3 Experimental design

Eighty one-day-old chicks were divided into four groups. In the first group each bird was infected by eyedrop (ED) with 50µl of M199 medium containing 3.38 $\log_{10}$ $TCID_{50}$ per 50µl, again divided into two,

in order to give an equal amounts of virus to each eye. The second and third groups were inoculated orally (Or) or subcutaneously (SC), with 0.3 ml of medium containing 3.38 $\log_{10}$ $TCID_{50}$ per 50µl per bird. The fourth group was left uninfected as controls

All four groups were kept in separate pens throughout the experiment.

6.4.4 Sampling

On days 3, 7, 14 and 21 pi, four birds from each experimental group were taken for sampling. From each bird, tears and blood were collected and then it was humanely killed. The HG and intestinal tract tissues were removed and samples collected for virus isolation and titration and the intestinal contents for antibody assay as described above. All samples were tested separately.

6.4.5 Virus isolation and titration

Virus titrations on intestinal and HG tissues were performed as described in Chapter 3.

6.4.6 Immunoglobulin assays

Samples of the intestinal contents, sera and tears were subjected to ELISA immunoglobulin assays. The antigen, buffers, monoclonal antibodies, conjugate and the procedure are described in Chapter 3. Results were analysed by the student t test using Starview, a statistical package for MacIntosh computers.

6.5 Results

6.5.1 Virus isolations and titrations

These are shown in Table 5.

Virus was detected in the HG only after ED infection on day three and at low titre. Samples from the other groups were negative.

Virus was present in all samples of gut in all groups on days 3, 7, and 14 pi. On each occasion, titres were of a similar order in each group, except that on day 3, the titre in the ED group was significantly higher than that in the SC group ($P<0.05$).
All tissues were negative on day 21 pi.

Table 5. Mean virus titres ±s.d. (log_{10} $TCID_{50}$ per 50µl) from the HG and the intestinal tract tissues of day-old chickens infected by eyedrop, orally or subcutaneously with reovirus strain R2

Days pi / Group	3		7		14		21	
	H.G	Gut	H.G	Gut	H.G	Gut	H.G	Gut
ED	1.82± 0.87	4.20± 0.41	-	2.39± 0.19	-	1.55± 0.33	-	-
OR	-	3.93± 0.37	-	2.87± 0.70	-	1.71± 0.43	-	-
SC	-	2.45± 0.74	-	3.67± 0.80	-	1.57± 0.22	-	-

-: negative

6.5.2 Immunoglobulin assays

6.5.2.1 IgA in tears

Reovirus-specific IgA in the tears first appeared on 7 day pi in all inoculated groups (Figure 9). OD levels continued to rise between days 14 and 21 pi when the experiment was terminated. Although the ED route induced a higher OD value than the other two on day 14

pi, there were no significant differences ($p<0.05$) between levels at the different times.

6.5.2.2 IgA in intestinal contents

There was no detectable reovirus-specific IgA in the intestinal contents in any of the infected groups during the course of this experiment (Figure 10).

6.5.2.3 IgA in sera

Very low levels of reovirus-specific IgA were detected on days 7, 14 and 21 pi after all routes of infection (Figure 11). However, the only sample significantly higher than the controls were those from the orally infected birds ($p<0.05$) on day 14.

6.5.2.4 IgG in tears

A rise in reovirus-specific IgG started on 7 dpi in the ED and SC groups and all increased through day 14 to 21 pi (Figure 12). Although the value for the subcutaneously-infected group was higher than the other routes on day 14 pi, there was no significant difference ($p<0.05$) between the infected groups at the different times.

6.5.2.5 IgG in intestinal contents

This immunoglobulin was not detected in the gut of any of the groups at any time after infection (not shown).

6.5.2.6 IgG in sera

Virus-specific IgG was first detected in the sera from day 7 pi and OD levels continued to rise up to day 21 pi (Figure 13). Results for all routes followed a similar pattern to IgG in the tears, including an increased level in the subcutaneously-infected group on day 14 pi.

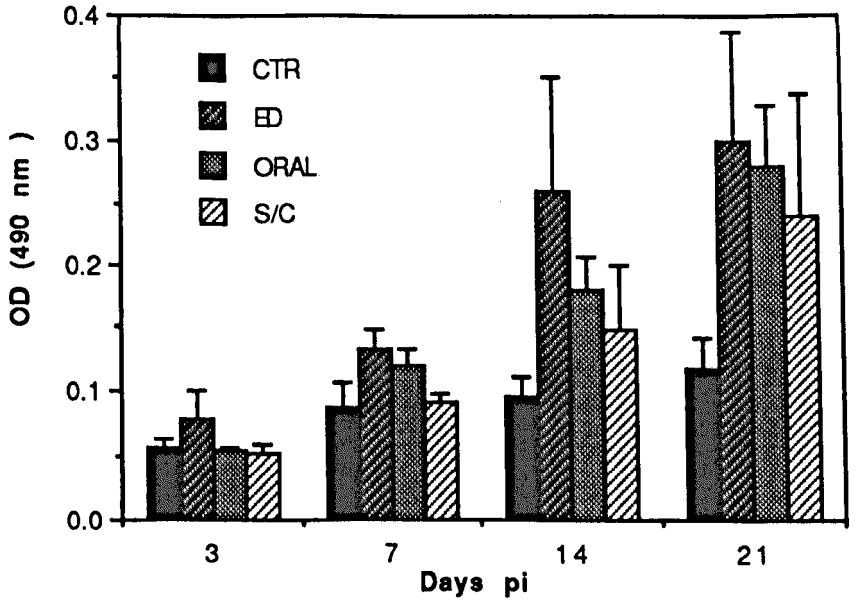

Figure 9. Reovirus-specific IgA in tears of chickens after eyedrop, oral or subcutaneous infection at day-old with reovirus.

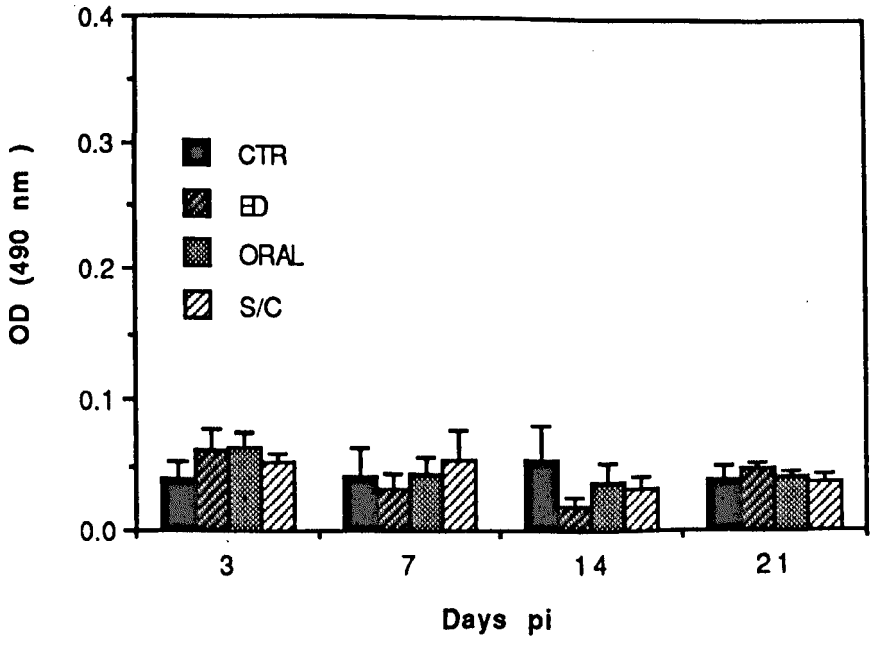

Figure 10. Reovirus-specific IgA in intestinal contents of chickens after eyedrop, oral or subcutaneous infection at day-old with reovirus.

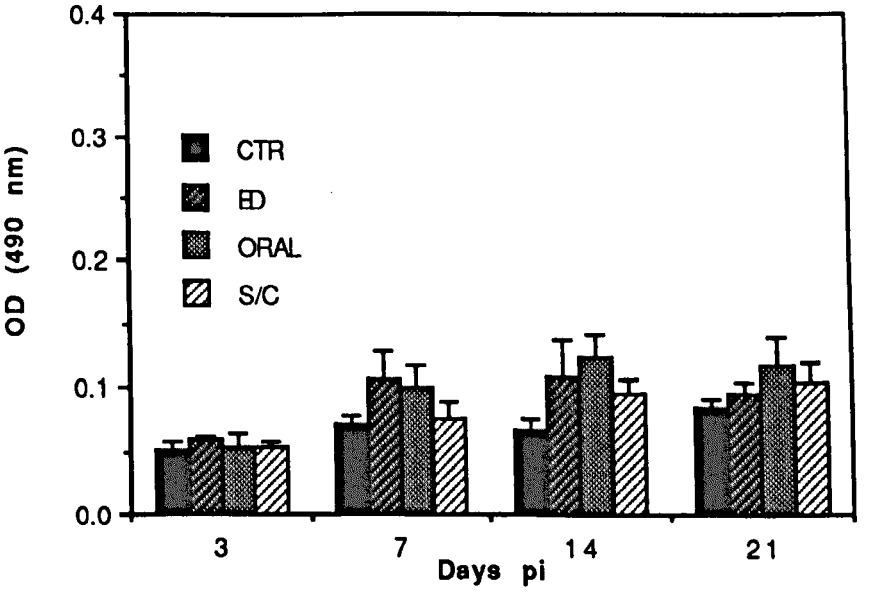

Figure 11. Reovirus-specific IgA in sera of chickens after eyedrop, oral or subcutaneous infection at day-old with reovirus.

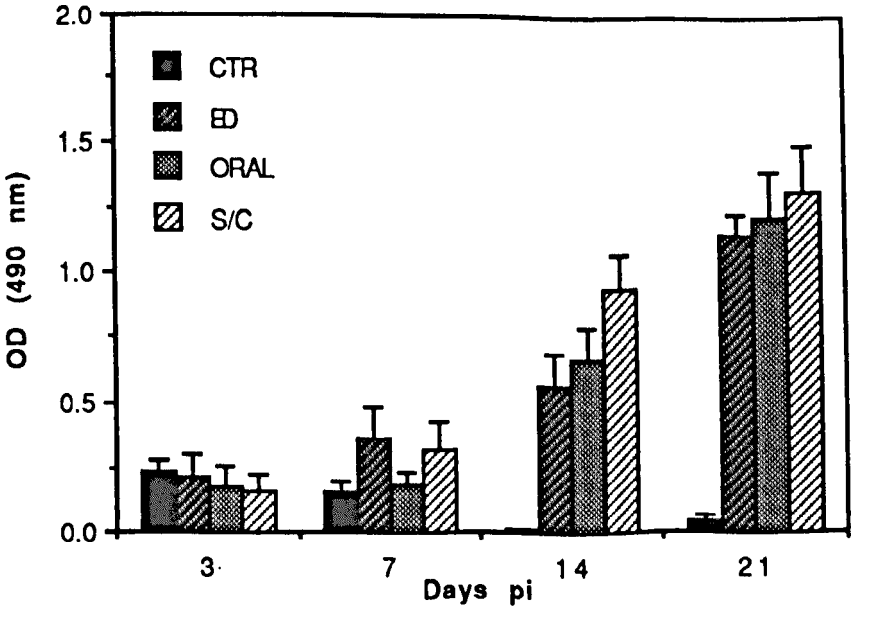

Figure 12. Reovirus-specific IgG in tears of chickens after eyedrop, oral or subcutaneous infection at day-old with reovirus.

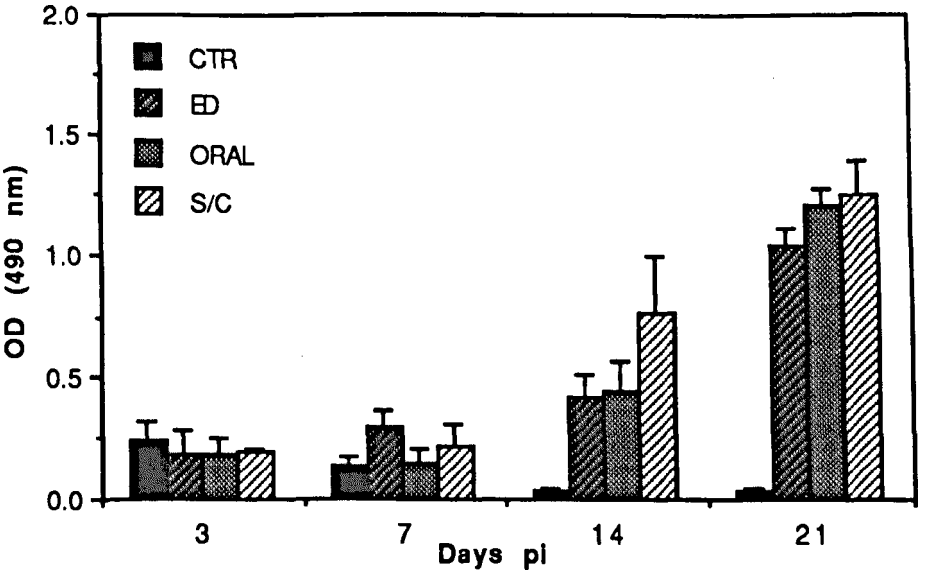

Figure 13. Reovirus-specific IgG in sera of chickens after eyedrop, oral or subcutaneous infection at day-old with reovirus.

6.6 Discussion

In Experiment 1, the isolation of reovirus from the HG of intra-ocularly infected birds, in albeit low but consistent titre from 3 to 5 days pi and its subsequent disappearance by day 10 indicated that the virus replicated in this tissue. Although avian reovirus has been extensively studied and shown to multiply in various tissues of the chicken (Menendez et al., 1975; Ellis et al., 1983; Kibenge et al., 1985; Jones et al., 1989), this is the first time the HG has been shown to support reovirus multiplication.

Virus isolation results for the intestinal tract tissues confirmed that the virus placed in the eyes, reached the gut, but it was short-lived, perhaps because the amount swallowed was small. The detection of reovirus-specific IgA in the tears after day 7 p.i. suggests that IgA is

likely to have participated of virus clearance from the HG by day 10 p.i.

Although it is difficult to directly compare the quantities of IgA in the tears to those in sera because of the difference in dilutions of test samples for the ELISA, the fact that IgA in the tears was detected in increasing amounts from day 7 p.i. up to the end of the experiment while that in sera was detected only between days 7 and 14 p.i. suggests that there was no transfer of IgA from HALT to the systemic circulation. Local production of IgA in the plasma cells of the Harderian gland has been shown to occur after infection with infectious brochitis virus (Davelaar et al., 1982) and Newcastle disease virus (Russell and Koch, 1993; Russell, 1993).

However, the detection of IgG in the tears and sera with a similar kinetic pattern indicates that lacrimal fluid IgG is likely to have originated from sera. The possibility of an active and selective transfer of IgG from serum to lacrimal fluid has been suggested by Davelaar et al. (1982), and this was later confirmed by Toro et al. (1993) and Suresh and Arp (1995).

Virus-neutralising antibodies in tears were detectable earlier and in substantially higher levels than those in sera. This may suggest that tears have more protective potential than sera, although in nature the two can not be treated separately. The higher titres in tears could be attributed in addition to IgA, IgG or IgM and perhaps other non-antibody factors associated with CMI. This is supported by the fact that the virus-neutralising antibodies appeared earlier than the virus-specific IgA.

The immunisation-challenge experiment was intended to investigate whether infection simulating vaccination by the ocular route could give protection against subsequent oral challenge by reovirus. When birds were immunised intra-ocularly or orally at day-old, and challenged at 38 days, no challenge virus was recovered from the gut tissues. However, the virus was recovered from the birds freshly

challenged birds at 38 days. This confirms that the infection at day-old by the ocular route did induce protection of the intestine. However, early oral infection did so also.

Chicks freshly-infected at day 38 pi produced detectable virus-specific IgA in the intestine on day 14 pc. However, in chicks initially infected orally at day-old and challenged at day 38 pi, virus-specific IgA in the intestine was not detectable. So it is likely that early infection induced protection but not mediated by IgA. Cell-mediated immunity has been suggested to play a role in controlling and clearing of many chicken enteric organisms including *Salmonella typhimurium* (Lessard et al., 1995), Eimeria spp (Lillehoj, 1987; Dominique et al., 1996), rotavirus (Myers and Schat, 1990) and in some respiratory viral infections including Newscastle disease virus (Marino and Hanson, 1987) and infectious bronchitis virus (Timms and Bracewell, 1981; Chubb et al., 1987; Cook et al., 1992). With regard to reovirus, Islam and Jones (1989) have reported marked delayed type hypersensitivity (DTH) in infected chicks when challenged with antigen at 6 weeks of age, but the exact role of this in protection is yet to be examined. The presence of IgA in the intestinal contents of the Ch group could be attributed to the older age at which these chicks were infected. It has been shown in Chapter 4 that virus-specific IgA is detectable in chicks infected after one week old. The absence of intestinal IgA in Or+Ch group could be either due to the young age at infection or to the fact that by the time of challenge virus replication did not occur. The same could have applied to groups ED and ED+Ch. Replication has been shown to be a vital determinant of intestinal response to rotavirus infection in mice (Shaw and Hempson, 1996).

There was no detectable virus-specific IgA in sera of chicks infected by any of the routes, with or without challenge. Serum IgA levels are known to be low and of short duration after infection (Mestecky and McGhee, 1987).

No reovirus-specific IgG was detected in the intestinal contents of chicks infected by either of the routes, whether challenged or not. Similar results were obtained in Chapter 4. It could be argued that the IgG in the gut is prone to proteolytic enzyme degradation, since it does not have secretory component like IgA (Watanabe and Kobayashi, 1974). It is also known that normally there is little IgG in the gut unless there is an inflammatory process which causes IgG to exude into the gut (Walker and Isselbacher, 1977).

Substantial amounts of virus-specific IgG in sera were detected in chicks infected by the ED or oral routes, whether challenged or not. The levels of virus-specific IgG of the Or+Ch and ED+Ch groups had a similar pattern from days 3 to 14 pc. This indicates that the two routes of inoculation if used are capable of inducing similar protection.

It can be clearly seen that both initial routes of infection induced an anmnestic response after challenge, a parameter which can be used in vaccination programmes.

Protection of young chicks against reovirus infection entails not only using an efficacious vaccine but also applying it by a method which is convenient and also prevent stress in chicken caused by injection. The second experiment compared three methods of administration of vaccines to young chicks but using a non-attenuated virus.

The production of local antibodies in the HALT and its importance in the defence against viral infection has been reported (Davelaar et al., 1982; Toro et al., 1991, 1993, 1994 and 1996; Russell, 1993; Russell and Koch, 1993). In the second experiment, virus-specific IgA in tears was detected from 7 to 21 days pi after all the three routes of infection. Although there was no significant difference between the routes, the ED route consistently induced more IgA than Or or SC infection. Perhaps surprisingly, the birds infected by the Or and SC routes also developed IgA in the tears even though virus apparently did not replicate in the HG after SC or Or infection. This result

confirms the finding by Obalda and Hanson (1989), who indicated that a portion of Newcastle disease virus inoculated by a parenteral route is capable of spreading to ocular tissues via the haematogenous route. On the other hand, the presence of reovirus-specific IgA in tears of parentally infected chickens could be a result of traffic between mucosal surfaces of cells destined to produce secretory antibody (Jayawardane et al., 1995).

No virus-specific IgA was detected in the intestinal contents of any of the birds following any of the three routes used. Furthermore, the amounts of IgA in the sera in all the three infected groups was very low. In a different study (Chapter 4 Part 1), it was shown that after oral infection with reovirus at day old, no virus-specific IgA was induced in the gut, but following infection at 7 or 21 days, the immunoglobulin appeared. As previously discussed, it is not clear whether the failure to detect IgA in gut was due to competitive binding or blocking of some kind by factors within the ELISA washes. Myers et al. (1989) have reported differences in the detection of intestinal antibodies due to competitive binding. It is speculated that adsorption of reovirus-specific IgA by reovirus antigens present in sera could reduce the detection of IgA.

No reovirus-specific IgA was detected in serum of chicks infected by either route, whatever age group. The reason for the absence of serum IgA is not clear but possible reasons were discussed in Chapter 4.

Virus-specific IgG in tears was detected from 7 to 21 dpi in all infected groups but with a significant difference ($p<0.05$) on 7 day pi, whereby the ED group had higher OD readings compared to the Or route. Since this occurs at this early stage, it is possible that IgG is produced by the HG earlier than systemically. Later, the IgG detected in tears could be a combination of that produced by the HG in addition to that transuding from the blood. The IgG in sera showed a similar pattern to that in the tears in all the infected groups. This

trend in similarity of the IgG kinetics in the tears and sera was also shown in the first experiment.

The results of this experiment show that for d.o chicks, there may not be any significant difference between these three routes of infection in inducing avian reovirus-specific IgA and IgG and this agrees with earlier findings by Islam et al. (1988) and Giambrone et al. (1991) using non-immunological parameters. However, if the coarse-spray method of application can be tailored to simulate eye-drop inoculation, then it could have some added advantages in that (a) it produces strong local immunity, (b) avoids handling the chicks (c) at this age it, ensures that each chick receives a sufficient dose of vaccine, unlike the drinking water route. Activation of the HG might also offer protection against reovirus which might be contracted by aerosol from dust. In addition to this, the quicker and higher virus-neutralising response in tears show that the HG has a role in reovirus immune responses.

CHAPTER 7

LACK OF CROSS-PROTECTION BY A TRYPSIN-SENSITIVE AVIAN REOVIRUS (TR1) AGAINST A TRYPSIN-RESISTANT STRAIN (R2) IN CHICKENS

CONTENTS PAGE

CHAPTER 7

LACK OF CROSS-PROTECTION BY A TRYPSIN-SENSITIVE AVIAN REOVIRUS (TR1) AGAINST A TRYPSIN-RESISTANT STRAIN (R2) IN CHICKENS

7.1 Introduction

Protection of chickens against viral tenosynovitis has been attempted by the use of passive immunity after vaccination of breeders (van der Heide, et al. 1976; Cessi and Lombardini 1975; Eidson et al., 1979 and 1985; van der Heide and Page, 1980; Wood and Thorton, 1981; Thorton and Wood, 1982; Jones and Nwajei, 1985; Wood et al 1986) or active immunity by early vaccination of chicks (Mandelli et al.,1969; van der Heide, 1973; Olson and Sahu, 1975; van der Heide et al., 1983; Eidson and Kleven 1983; Giambrone and Closer 1989; Giambrone and Hathcock, 1991).

Despite the longtime use of breeder vaccination, viral tenosynovitis still occurs with its consequential economic losses. The occurrence of viral tenosynovitis in the progeny after breeder vaccination could possibly be due to: (a) improper vaccination of the pullets (b) decreased maternal immunity in older birds, and (c) the necessity to induce cell-mediated immunity which passive immunity does not cause (Giambrone and Hathock, 1991)

Although most avian reoviruses are antigenically related and show considerable *in vitro* cross-reactivity (Kawamura and Tsubahara 1966; Olson and Weiss 1972; Sahu and Olson 1975; Sahu et al 1979; Wood et al., 1980; Roberson and Wilcox 1984), they show a great degree of lack of cross-protection. It is of interest to note that vaccination based on strain S 1133, the one commonly used to vaccinate against field avian reoviruses is also

one in which lack of cross-protection has been demonstrated (Springer, et al., 1970, 1982; Rau et al., 1980; Wood and Thorton, 1981; Thorton and Wood, 1982; Wood et al., 1986). Incomplete protection has also been described with the Japanese strain 58-132 (Takase et al., 1996).

To overcome this narrow range of cross-protection in young stock, another vaccine derived from strain UMI-203 (Haffer 1984) was developed and it was shown to have a broader antigenicity than S1133 (Townsend et al., 1976).

Among breeder vaccinations, the problems associated with the relatively narrow range of protection conferred to chickens by their use, compared to the wide range of antigenic types of avian reovirus ubiquitous in the chicken population, lead to the development of vaccines with broader antigenicity. Two such vaccines were made available for breeder vaccination in the USA (Robertson and Wilcox 1986).

While the lack of cross-protection by some strains is still unresolved, the fact that a number of avian reoviruses (including S1133) have recently been found to be trypsin-sensitive (Jones et al., 1996), raises even more concern on their suitability for use as vaccines.
In this study a trypsin-sensitive avian reovirus strain TR1 already described in Chapter 3., is investigated to see whether it can cross-protect against a trypsin-resistant strain R2.

7.2 Materials and methods

7.2.1 Chickens

SPF WLH chickens from the department's flock were used. They were given water and food *ad libitum*.

7.2.2 Viruses

The trypsin-sensitive strain TR1 and a trypsin-resistant strain R2 already described in (Chapter 3) were used. Using polyclonal chicken sera, antisera to R2 neutralises TR1 at a 4-fold lower dilution than R2. However, TR1 antisera neutralises both TR1 and R2 equally strongly (Al-Mufarrej, 1994). Recent results (Jones personal communication) suggest that these two strains are closely related but not identical. The inoculum of each virus was 0.3 ml of medium M199 containing 3.18 $\log_{10}$ $TCID_{50}$ per 50µl and was given by the oral route.

7.2.3 Experimental design

The chicks were kept in strict isolation until they were three weeks old when the experiment started (Table 1). Initially, chickens were divided into three groups, two of which comprised 40 and 28 birds each,while another contained 16 as controls. The group of 40 was divided into a subgroup of 12 and left uninfected (-/R2) while that of 28 was infected orally with reovirus strain R2. Out of these 28, four were for pre-challenge sampling, 12 were to be left un-challenged (R2/-) and the other 12 were to be challenged (R2/R2) The other large group was infected orally with TR1. From this group, four birds were for pre-challenge sampling, 12 were to be challenged (TR1/R2) and the rest left unchallenged (TR1/-). For the purposes of this experiment the initial infection with R2 or TR1 are refered to as 'immunisations'. The control group was uninfected throughout the experiment. All chickens were kept for four weeks before some were challenged with R2.

Group	Treatment	
	Immunised at 3 weeks	Challenge at 7 weeks
R2/R2	R2	R2
R2/-	R2	-
-/R2	-	R2
TR1/R2	TR1	R2
TR1/-	TR1	-
CTR	-	-

Table 1 shows experimental treatments to various groups.

7.2.4 Sampling

On days 2, 4 and 14 post-challenge four birds from each group were sampled. Tears were induced and collected for reovirus-specific IgA and IgG assay as already described (see Chapter 6). Blood was collected for virus-specific IgG assay. The birds were then killed humanely and the intestinal tracts removed. For each bird, representative samples of the duodenum, jejunum, ileum and caecum were collected and pooled for virus isolation and titration. From these gut pieces samples of the intestinal contents were gently squeezed out, pooled and immediately processed as already described in Chapter 4. Samples of the joints, spleen and liver were collected for virus isolation and titration as already described in Chapter 3.

Expression of results

All statistical analyses were subjected to the t test in Starview, A statistical package for Macintosh computer.

7.3 Results

7.3.1 Virus isolations

Pre-challenge

All the intestinal and joint tissues of the pre-challenge chickens, whether immunised with R2 or TR1 strain, were negative at 7 weeks old (4 weeks p.i).

Post-challenge

Table 2 summarizes the virus isolation results of the liver, spleen, hock joint and the intestinal tissues of chickens immunised at 3 weeks old with either TR1 or R2 and then challenged with R2 4 weeks later.

Gut

All primarily uninfected birds but challenged (-/R2), were positive for virus on days 2 and 4 pc but negative on day14 pc (Table 2).

After challenge, no virus was recovered from the chickens which had been immunised with strain R2 (R2/R2). However all birds which had been immunised with strain TR1 (TR1/R2), were positive on days 2 and 4 pc with R2. but negative on day 14 pc.

Liver

All chickens which had been immunised either with TR1 (TR1/R2) or R2 (R2/R2) were negative for virus. Virus was isolated only from the previously uninfected but challenged group (-/R2) and even then only on day 4 pc.

Spleen

Virus was isolated only from 2/4 of spleens of the chickens which had been immunised with TR1 on day 4 pc only.

Joint

All the hock joints examined were negative for virus.

Table. 2. Virus isolation results from the gut, liver, spleen and joint of chickens either immunised with TR1 or R2 at 3 weeks of age and challenged with R2 4 weeks later.
4/41: positive/number sampled; n/d: not done

Group	Tissue	Days post challenge			Total Isolations
		2	4	14	
R2/R2	Gut	0/4	0/4	0/4	0
	Liver	0/4	0/4	0/4	0
	Spleen	0/4	0/4	0/4	0
	Joint	n/d	n/d	0/4	0
-/R2	Gut	4/4	4/4	0/4	8
	Liver	0/4	2/4	0/4	2
	Spleen	0/4	0/4	0/4	0
	Joint	n/d	n/d	0/4	0
TR1/R2	Gut	4/4	4/4	0/4	8
	Liver	0/4	0/4	0/4	0
	Spleen	0/4	2/4	0/4	2
	Joint	n/d	n/d	0/4	0

R2/-, TR1/- and CTR were negative throughout.

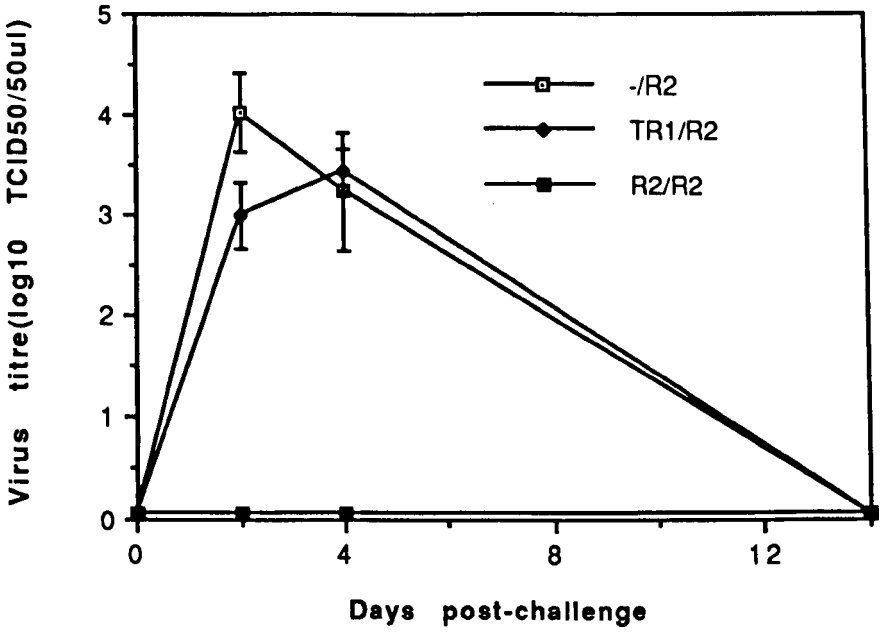

Figure 1. Mean virus titres post-challenge in the intestinal tissue of chickens infected at 3wk and challenged at 7wks.

7.3.2 Immunoglobulin assays

Reovirus-specific IgA in tears and intestinal contents, are shown in Figure 2 and 3 and IgG in tears, intestinal contents and sera in Figures 4, 5 and 6 respectively.

7.3.2.1 IgA in tears

At the time of challenge, when the birds were 7 weeks old, there was detectable virus-specific IgA in the tears of chickens previously immunised when 3 weeks with either R2 or TR1, but with no significant difference between them ($p<0.05$) (Figure 2). After challenge, these birds had increased IgA responses, but still without any significant differences between those immunised with R2 or TR1.

Predictably, the -/R2 group responded to the challenge virus as evidenced by the appearance of virus-specific IgA by day 14 pc. However, there was no significant difference between this group and those immunised with either TR1 or R2. Looking at the rate of

response after challenge, the group immunised with R2 had a steeper response than those given TR1, and the level of IgA was significantly higher on day 14 pc ($p<0.05$).

7.3.2.2 IgA in intestinal contents

Pre-challenge sampling shows that no reovirus-specific IgA was induced in chickens immunized with TR1 (Figure 3). However those immunised with R2 had a substantial virus-specific IgA response.

After challenge, there was a slight decline in the IgA of the chickens which had earlier been immunised with R2, but it was still significantly higher than that in the controls ($p<0.05$). The chickens originally immunised with TR1, responded to the challenge virus (R2), and produced a high level of virus-specific IgA by day 14 pc. The non-immunised group which was freshly infected at 7 weeks (-/R2), produced some IgA but of modest quantity, although it was comparable to that of the group originally immunised with R2 at 3 weeks old. It is noteworthy that although the chickens immunised with TR1 had no detectable virus-specific IgA, on challenge, they responded with a much higher OD level than those freshly infected at 7 weeks (-/R2).

7.3.2.3 IgG in tears

The chickens which had been immunised with either strain R2 or TR1 at 3 weeks old showed high levels of residual virus-specific IgG at 7 weeks pi (pre-challenge) (Figure 4). After challenge, both groups showed a identical but modest increases in OD levels by day 14 pc. The birds infected for the first time at 7 weeks (R2(C) also responded by day 14 pc.

7.3.2.4 IgG in intestinal contents

No significant levels of virus-specific IgG were detected in the intestinal contents of all chickens infected with either TR1 or R2 (Figure 5)

7.3.2.5 IgG in sera

Virus-specific IgG in sera followed a similar trend to that in the tears, as shown in Figure 6.

Immunisation with strain R2 or TR1 caused a response which was still detectable by 7 weeks pi, which increased after challenge. By day 14 pc, those infected for the first time at 7 weeks produced IgG which was comparable to that in the groups that had been immunised and challenged.

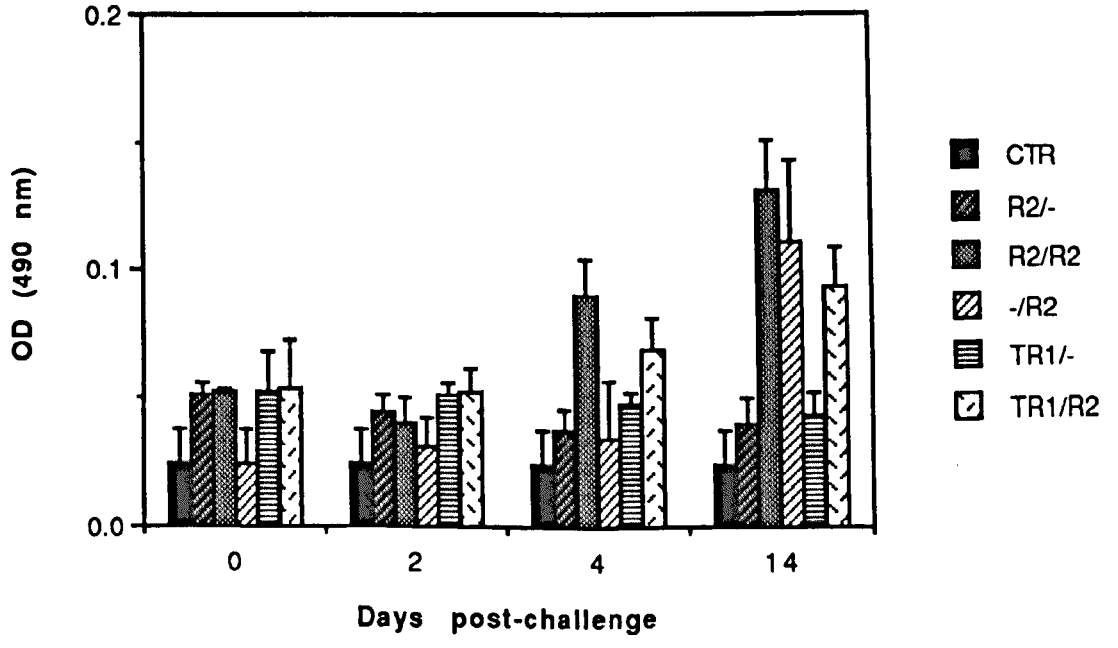

Figure 2. Reovirus-specific IgA (OD mean±sd) in tears of chickens infected with either TR1, R2 or non-infected at 3 weeks of age and then challenged with R2 at 7 weeks old.

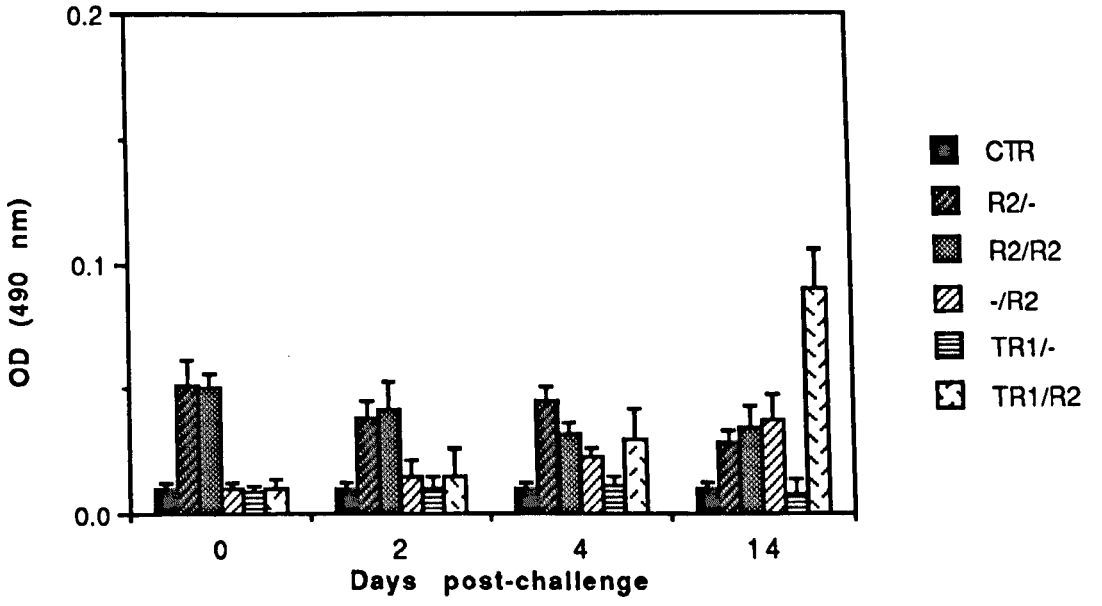

Figure 3. Reovirus-specific IgA (OD mean±sd) in the intestinal contents of chickens infected with either TR1, R2 or non-infected at 3 weeks of age and then challenged with R2 at 7 weeks old.

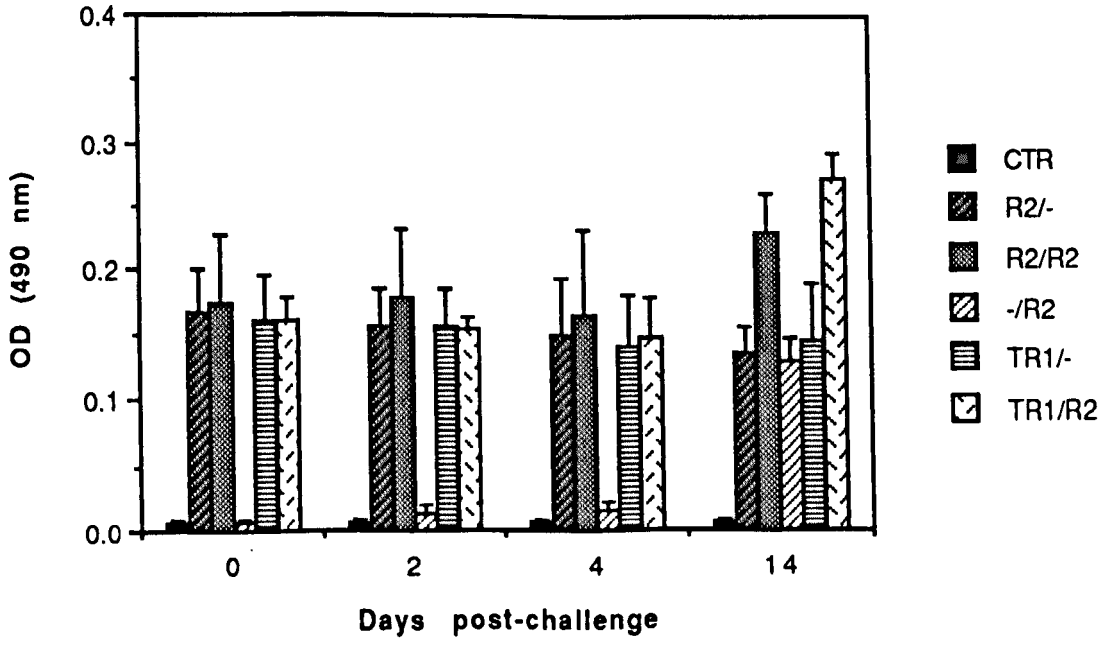

Figure 4. Reovirus-specific IgG (OD mean±sd) in tears of chickens infected with either TR1, R2 or non-infected at 3 weeks of age and then challenged with R2 at 7 weeks old.

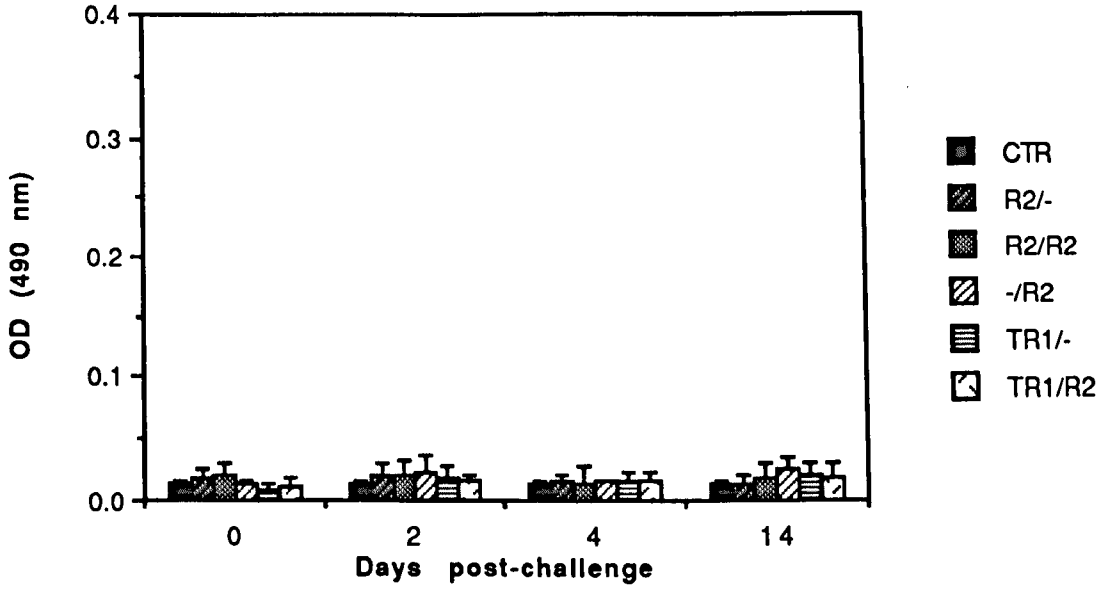

Figure 5. Reovirus-specific IgG (OD mean±sd) in intestinal contents of chickens infected with either TR1, R2 or non-infected at 3 weeks of age and then challenged with R2 at 7 weeks old.

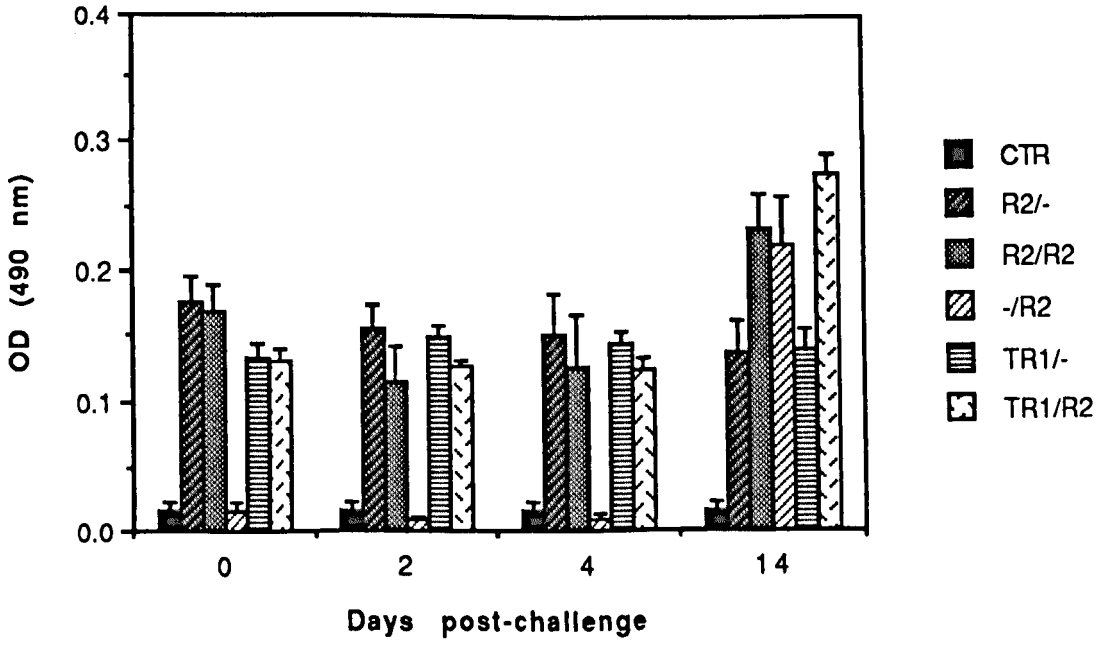

Figure 6. Reovirus-specific IgG (OD mean±sd) in sera of chickens infected with either TR1, R2 or non-infected at 3 weeks of age and then challenged with R2 at 7 weeks old.

7.4 Discussion

Although avian reovirus vaccines have been in use for over two decades, there is still considerable difficulty encountered in attempting to provide complete protection by use of either homologous or heterologous viruses (Rau et al 1980; Giambrone et al. 1991). This is in part due to the relatively poor immunogenicity of these viruses as indicated by lack of complete neutralisation *in vitro* between antigen and antibody (Kawamura et al. 1965; Sahu and Olson 1975; Wood et al. 1980; Robertson and Wilcox 1984), or lack of cross-protection *in vivo*. Efforts therefore continue to be devoted to a search for better vaccines and some of the parameters which may require testing for, is their ability to cross-protect against heterologous strains.

In this study non-attenuated strains of a trypsin-sensitive reovirus TR1 and a trypsin-resistant R2 were used to establish whether they could equally protect against subsquent challenge by R2. Virus reisolation and virus-specific immune responses were used to monitor protection. Challenge by TR1 was not attempted because it would not replicate in the gut (see Chapter 4). Thus efficacy of immunity, as judged by virus isolation after challenge could not have been monitored.

In chickens immunised with R2 when 3 weeks old, no residual virus could be isolated from the gut and joint tissues prior to challenge at 7 weeks of age. This results was expected in the case of the gut tissues, because reovirus is known to be relatively short lived in this site in chicks infected at 3 weeks (see Chapter 4). However, the lack of recovery of virus from the joints was unexpected, but this was possibly also due to the older age of the birds at infection and the relatively lower dose of inoculum used. This age-related resistance of chickens to avian reovirus infection was first reported by Kerr and Olson (1964) and conclusively demonstrated by Jones and Georgiou (1984).

Immunisation with R2 clearly protected birds against challenge with the same virus, since no challenge virus was recovered from the tissues. At the same time, challenge virus was recovered from tissues of those not previously immunised. TR1 immunisation did not, however, protect against challenge with R2, since the pattern of virus isolation from tissues resembled that in the -/R2 group.

Furthermore, there was, although only once, reisolation of virus from the spleen of the TR1 group. The spleen is a highly active organ in the immune function of vertebrates and if there had been any protection by TR1, then at least this organ might have been expected to be the least to lack protection. Since the spleen was not sampled at pre-challenge, it could be argued that TR1 might have persisted in the spleen. However, such a deduction might be unjustified, considering the age of chickens used, route and dose used especially with a trypsin-sensitive strain. Roessler and Rosenberger (1989) managed to reisolate reovirus from spleen of chicken after infection with strains of high or intermediate pathogenicity strains but not with a mild one, and they used the intratracheal route.

Examination of reovirus-specific IgA in tears indicated that there was a response induced by both TR1 and R2 when chickens were immunised at 3 weeks old, which persisted until challenge. This increased after challenge, due to the anamnestic response. However the rise after challenge was higher in the R2-immunised group than in those immunised with TR1, although the difference was not statistically significant. This may have reflected the partial relationship between these two viruses.

In the gut, chickens immunised with TR1 at 3 weeks did not show any reovirus-specific IgA in the intestinal washes at the pre-challenge time, while those infected with R2 had significant ($p<0.05$) levels. The trypsin sensitivity of the reovirus strain TR1 is known to be responsible for a markedly reduced level or absence of replication in the enteric tissues compared to the trypsin-resistant strain R2 (Jones et al., 1994) and in Chapter 4, it has been shown that at the titre used, this virus does not replicate

in the gut. Replication of virus, especially in the gut mucosa has been shown to be an important determinant of mucosal IgA responses in rotavirus infection of mice (Shaw and Hempson 1996). The significantly high levels of IgA in the TR1/R2 birds on day 14 pc, indicate that the challenge virus induced a response and since there was no residual IgA at the time of challenge, this virus was not neutralised.

Chickens that had been immunised with R2 at 3 weeks of age and showed an IgA response in the gut, did not appear to elicit any increase in virus-specific IgA after challenge. The cause of this is not clear, but it could be attributed to neutralisation of virus by IgA. Such antibody-antigen binding results into the formation of immune complexes in the intestines which may facilitate other protective mechanisms (Davelaar et al., 1982; Walker and Isselbacher 1977).

No significant levels of virus-specific IgG were detected in the intestinal washes of chickens immunised with either TR1 or R2. Similar results were obtained by Myers and Schat, (1990) who used an identical ELISA assay to ours in rotavirus studies. In mammalian studies, this lack of detection of IgG in Isotype-specific ELISAs has been frequently encountered and it is attributed to competition either between isotypes or within a particular isotype (van Zaane and Ijzerman 1984; Kimman et al., 1987). Another possible cause could be the degradation of IgG by proteolytic enzymes in the gut especially when samples are kept for long before processing (Porter and Holt, 1992).

Reovirus-specific IgG in tears and sera had virtually identical patterns of response after early infection with either TR1 or R2. This similarity could further substantiate the findings by Toro et al. (1993) that sytemic IgG is actively transuded into tears. The birds immunised at 3 weeks still had substantial amounts of virus-specific IgG in tears by the time they were challenged and the level of IgG increased after challenge. However, the increase was not dramatic and in some cases it was not significantly higher than the levels before challenge. This modest increase (anamnestic

response) after challenge could be a characteristic of the systemic reovirus immune response, since it was also demonstrated by Al-Afaleq and Jones (1994) and they attributed it to the poor immunogenicity of the virus.

This study clearly confirmed that although there was no detectable TR1 replication in the intestinal tract and subsquently no intestinal IgA production, there was however, virus-specific IgA in the tears of TR1 infected chickens. This suggests that there could be a means by which TR1 gets to the eye tissues and especially the Harderian gland in order to stimulate production of the detected IgA. However, the presence of this IgA (and indeed IgG) in the tears did not prevent the challenge virus replicating in the gut, which further indicates that this IgA is produced locally and acts locally as well.

A key factor in this study is the relationship between the two reovirus strains. There appears to be a partial but not complete antigenic relationship, and the immune responses have illustrated this (see later). However, it seems more likely that the failure of TR1 to protect against R2 challenge is related to its inability to replicate in the intestine and induce virus-specific IgA and perhaps other important immune responses.

Further work is necessary to establish whether R2 in attenuated form could induce protection against heterologous challenges when applied either actively at day-old, or passively via parental immunisation.

CHAPTER 8

IN VITRO STUDIES ON REOVIRUS REPLICATION AND IMMUNE RESPONSES IN REGIONS OF THE GUT

CONTENTS PAGE

CHAPTER 8

IN VITRO STUDIES ON REOVIRUS REPLICATION AND IMMUNE RESPONSES IN REGIONS OF THE GUT

In addition to previous reports (Menendez et al., 1975; Ellis et al., 1983; Jones et al., 1986), it was shown in Chapter 4. that avian reoviruses are able to replicate in the intestinal tract of chicks. In Experiment 1 of this Chapter, *in vitro* techniques are used to study the susceptibility of different regions of the intestine by inoculating explants with graded doses of virus. The second experiment examines the production of local reovirus-specific IgA and IgG in explants of intestinal tissues.

EXPERIMENT 1. REOVIRUS MULTIPLICATION IN VARIOUS REGIONS OF THE CHICK INTESTINE AFTER INFECTION WITH GRADED DOSES OF VIRUS

8.1 Introduction

Avian reoviruses are primarily enteric viruses and have been shown to replicate in the gut to high titres after oral infection (Menendez et al., 1975; Ellis et al., 1983; Jones et al., 1986). *In vivo* avian reovirus R2, has been shown to replicate mainly in the duodenum and jejunum (Jones et al., 1989). This is perhaps surprising, because virus might have been expected to multiply to a higher titre in the posterior regions of the gut where environmental conditions are less harsh, since the enzymes and acids are secreted in the anterior portions of the gut.

In Chapter 4 it was found that when chicks were infected orally with 3.5 $\log_{10}$ $TCID_{50}$ per 50µl of reovirus strain R2, virus was recovered from all parts of the gut, but the highest isolation indices were recorded in the caecum, rectum, duodenum and ileum and the lowest in the jejunum. This would indicate that some of the virus recovered in the posterior part of the gut could have been produced partly in the anterior regions, but was passed on to the caecum and rectum by peristalsis.

Because most of the previous reports on avian reovirus replication in the gut have used *in vivo* experiments, we chose an *in vitro* procedure, since it enables the handling of specific regions of the gut to be examined separately and the interference of systemic effects minimised. The effects of virus shedding from the anterior part to the posterior is also avoided. Similar organ culture methods have been used for infectious bronchitis (Colwell and Lukert, 1969; Johnson et al., 1969; Darbyshire et al., 1978; Pradhan et al., 1983), adenovirus (Georgiou et al., 1983) and reovirus (Afaleq and Jones, 1989).

The purpose of this experiment was to show how different tissues of the gut support avian reovirus replication *in vitro* and where possible, relate it to the intestinal immune response in the second experiment.

8.2 Materials and methods

8.2.1 Chickens

Ten 4-week old SPF chickens were used which had been kept in isolation and given water and food *ad libitum*.

8.2.2 Virus

Arthropic avian reovirus R2, already described in Chapter 3 was used.

8.2.3 Intestinal organ cultures

8.2.3.1 Preparation of the intestinal organ cultures

The birds were deprived of food for three hours, being given only water before they were humanely killed. The alimentary tract was removed between the gizzard-duodenal junction and the posterior rectum. Five cm lengths each of duodenum, jejunum, ileum, caecum and rectum were collected from each of the chickens. Similar tissues of the gut from each bird were pooled in one petri dish. These were opened with scissors and washed with PBS (with x10 concentrations of penicillin and streptomycin-see Appendix A). Small pieces of approximately 3mm square were cut using a scapel blade and washed twice in pre-warmed (37^0C) PBS in separated petri dishes for each tissue of the gut. After a second wash in PBS, the tissues were transferred to another petri dish containing 10 ml of pre-warmed MEM medium (Eagles Minimum Essential Medium containing Earles salts and glutamine with 250 units/ml penicillin and 0.25 mg/ml streptomycin) (see Appendix A).

Two pieces each of the duodenum, jejunum, ileum, caecum and rectum were put in each of ten roller-tubes. Twenty organ cultures from each intestinal region were collected, giving a total of 100 tissues from the five chickens for each virus titre.

8.2.4 Experimental design

The virus used was in doses of 2.0, 3.0 or 4.0 $\log_{10}$ $TCID_{50}$ per 50µl in MEM medium. Each tube containing two tissue pieces was inoculated with 0.2ml of MEM medium containing the virus dilution and there were five replicates of each. A gentle mix of the tissue in the suspension of virus was made in order to ensure good contact of virus with the tissue. All the tubes were placed in roller drums and incubated for 1 hour at 37^0C to allow for virus adsorption.

After adsorption, the inoculum was removed with a pasteur pipette and the tissues were washed three times with 1ml of pre-warmed PBS, to ensure that the only virus remaining was that adsorbed. The controls were similarly treated but instead of virus, 0.2 ml of MEM was added. After washing, 0.5 ml of MEM medium was added to each tube and they were kept at 37^0C in the roller drum.

Additional control tubes containing 0.2 ml of virus inoculum but without intestinal tissues were also set up in order to ensure that the washing after incubation was sufficient to clean the tubes free of all virus and ensure that virus detected was only that released from the tissues.

After each of 1, 2, 3, 4, and 5 days of incubation, a pool of the medium from the five tubes of like tissues with the same inoculum was collected into a total of 2.5 ml for each sampling time. Fresh pre-warmed MEM medium was added on each occasion to the tissues which were re-incubated.

8.2.5 Virus titration in the medium

Virus titration was performed on each of the pools of medium following the method described in Chapter 3.

8.3 Results

8.3.1 Virus titrations

These are shown in Tables 1, 2, and 3 and Figures 1, 2, 3, 4 and 5.

Duodenum

After the 2.0 log_{10} inoculum, virus replication in the duodenum was detected on day 3 pi and it continued up to day 5 pi (Figure 1). With 3.0 log_{10} virus replication was detected a day earlier than with 2.0 log_{10} and by day 5 the titre was 3.83 log_{10}. Likewise with the highest dose of 4.0 log_{10}, replication commenced on day 1 and quickly rose through 3.83 log_{10} to reach the highest titre of 4.0 log_{10}.

Jejunum

With 2.0 log_{10} dose, no virus replication was detected in the jejunal organ cultures (Figure 2). Using the 3.0 log_{10} inoculum, replication was detected from day 2 pi and continued to day 5 pi. After the 4.0 log_{10} inoculum, replication was detected by day 1 pi and attained the highest titre by day 3 pi which was maintained up to day 5 pi.

Ileum

No virus replication was detected when a 2.0 log_{10} inoculum was used (Figure 3). With a 3.0 log_{10} inoculum, there was virus replication as early as day 1 pi and by day 5 pi it had reached a titre of 2.63 log_{10}. With the 4.0 log_{10} inoculum, replication was higher than in the dose of 3.0 log_{10} at all days.

Caecum

No virus was detected in the organ cultures of the caecum when infected with the 2.0 or 3.0 log_{10} inocula (Figure 4). However, with

a higher dose of 4.0 log_{10}, replication was detected at day 1 pi and rose to reach the highest titre by day 3 pi.

Rectum

No virus was detected when the 2.0 log_{10} inoculum was used (Figure 5). With the 3.0 log_{10} replication was detected at day 3 and 4 pi only and the titres were less than 2.0 log_{10}. With the 4.0 log_{10} inoculum, virus replication was detected by day 1 pi and replication continued up to day 5 pi.

Table 1. Virus titres in various tissues of the intestine inoculated with avian reovirus strain R2 at a dose of 2.0 $\log_{10}$ $TCID_{50}$ per 50µl

Days pi Tissue	1	2	3	4	5
Duodenum	-	-	1.5	1.63	2.5
Jejunum	-	-	-	-	-
Ileum	-	-	-	-	-
Caecum	-	-	-	-	-
Rectum	-	-	-	-	-

Table 2. Virus titres in various tissues of the intestine inoculated with avian reovirus strain R2 at a dose of 3.0 $\log_{10}$ $TCID_{50}$ per 50µl

Days pi Tissue	1	2	3	4	5
Duodenum	-	1.32	1.63	1.5	3.83
Jejunum	-	1.32	2.5	2.5	2.83
Ileum	1.38	1.5	1.83	1.5	2.63
Caecum	-	-	-	-	-
Rectum	-	-	1.32	1.5	-

Table 3. Virus titres in various tissues of the intestine inoculated with avian reovirus strain R2 at a dose of 4.0 $\log_{10}$ $TCID_{50}$ per 50µl

Days pi Tissue	1	2	3	4	5
Duodenum	1.32	2.38	3.83	4.0	4.0
Jejunum	2.0	3.38	4.0	4.0	4.0
Ileum	1.5	2.63	2.63	4.0	4.0
Caecum	2.38	2.83	3.5	3.63	3.63
Rectum	2.5	2.63	3.5	4.0	3.83

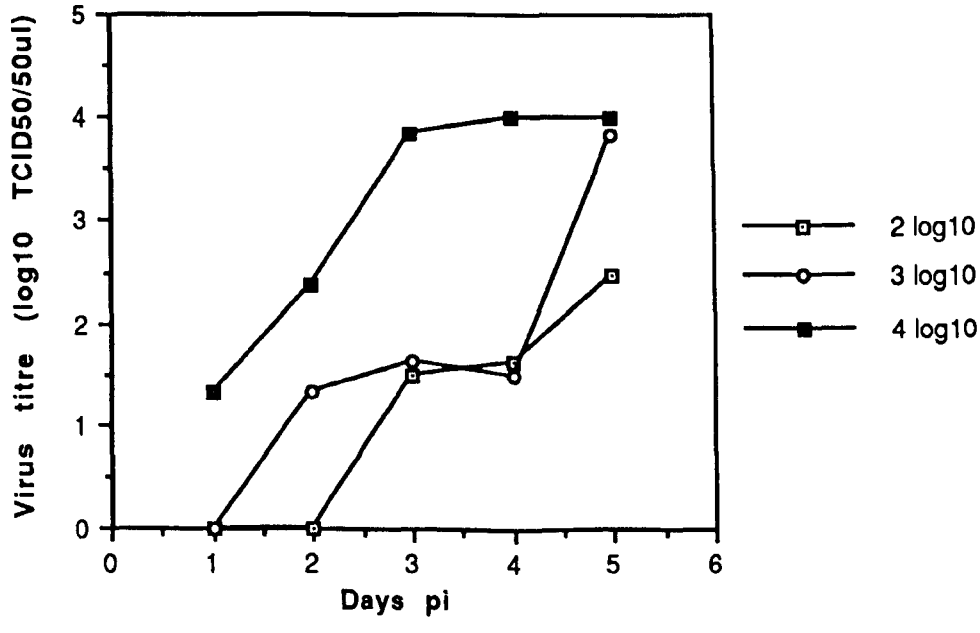

Figure 1. Virus titres in explants of duodenum from 4-week old chicks inoculated with 2.0, 3.0 or 4.0 $\log_{10}$ $TCID_{50}$/50µl of reovirus strain R2.

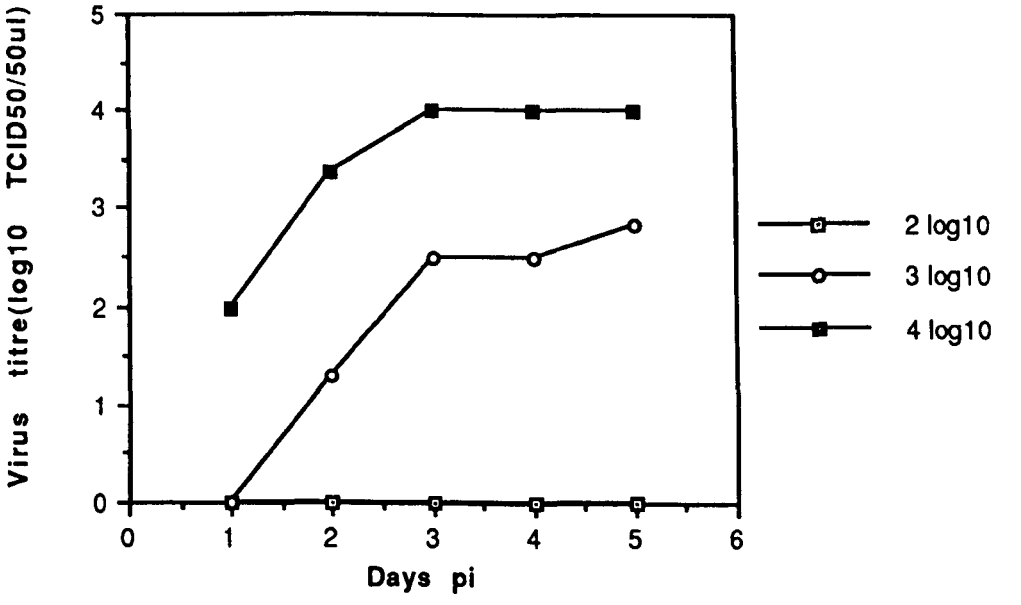

Figure 2. Virus titres in explants of jejunum from 4-week old chicks inoculated with 2.0, 3.0 or 4.0 $\log_{10}$ $TCID_{50}$/50µl of reovirus strain R2.

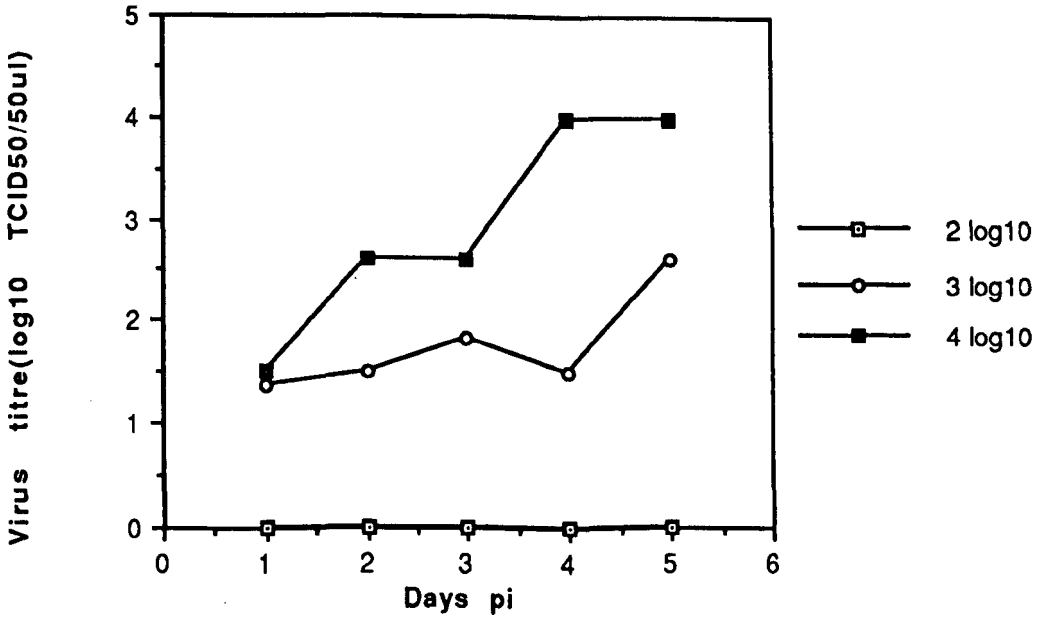

Figure 3. Virus titres in explants of ileum from 4-week old chicks inoculated with 2.0, 3.0 or 4.0 $\log_{10}$ $TCID_{50}$/50µl of reovirus strain R2.

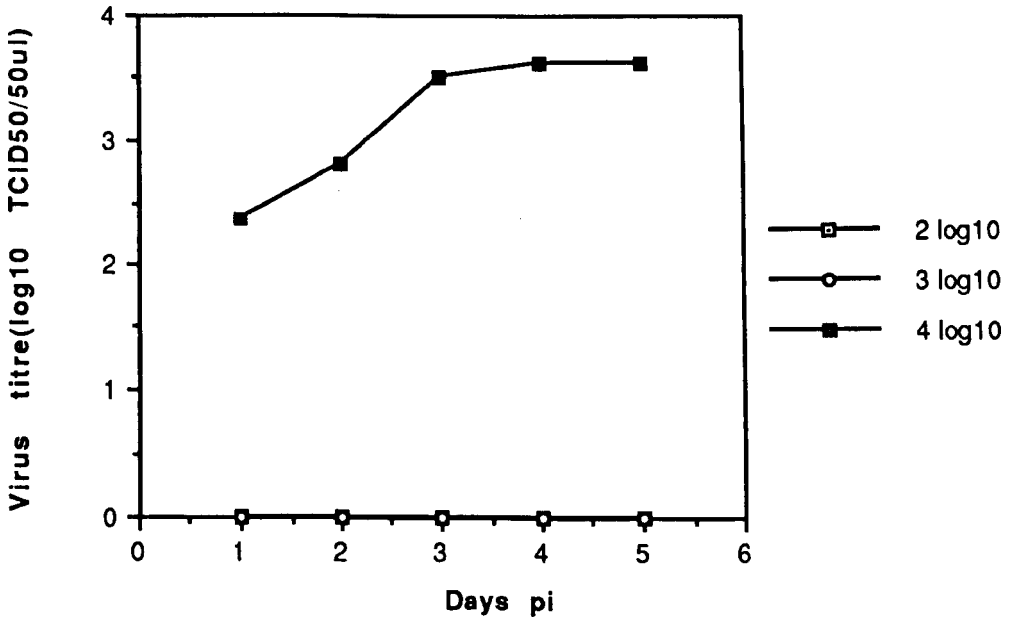

Figure 4. Virus titres in explants of caecum from 4-week old chicks inoculated with 2.0, 3.0 or 4.0 $\log_{10}$ $TCID_{50}$/50µl of reovirus strain R2.

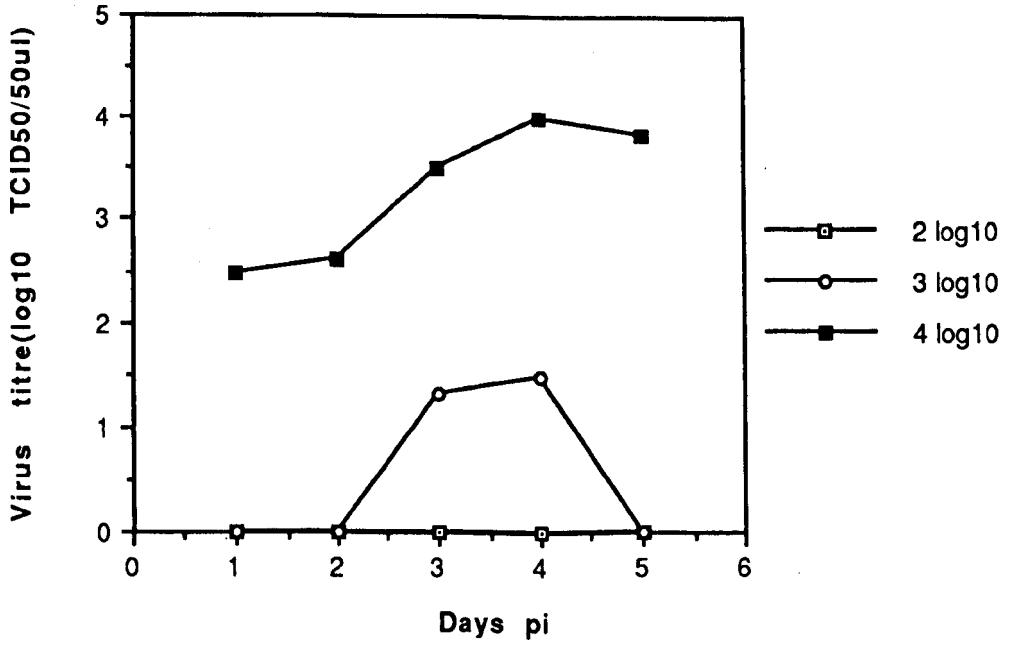

Figure 5. Virus titres in explants of rectum from 4-week old chicks inoculated with 2.0, 3.0 or 4.0 $\log_{10}$ $TCID_{50}$/50µl of reovirus strain R2.

EXPERIMENT 2. LOCAL ANTIBODY PRODUCTION IN VARIOUS REGIONS OF THE CHICK INTESTINE AFTER INFECTION WITH REOVIRUS

8.4 Introduction

The mucosal surfaces constitute important portals of entry for many infectious organisms in the chicken, including *E.coli* (Morris and Sojka, 1985), infectious bronchitis (Hofstad, 1984), *Eimeria* species (Rose, 1987; Fernando et al., 1987) and rotavirus (Myers and Schat, 1991).

As in mammals, the mucosal immune system is well developed in chickens and among its protective activities against foreign antigens is the production and secretion of local immunoglobulins. In the gut mucosal system, IgA is the major immunoglobulin found in secretions (Lebacq-Verheyden et al., 1972). The production of local antibodies in the intestine of chickens after infection has been reported among others for coccidiosis (Orlans and Rose, 1972), avian reovirus (Watanabe et al., 1975), Newcastle disease virus (Lee and Hanson, 1975; Jayawardane and Spradbrow, 1995), *E.coli* (Parry et al., 1977) and rotavirus (Myers and Schat, 1990).

Avian reoviruses are principally enterotropic and replicate in the intestine (Menendez et al., 1975; Ellis et al., 1983; Jones et al., 1986) but experimentally, this replication is relatively short lived. Factors which may contribute to this limited replication could be age (Jones and Georgiou, 1984; Kataria et al., 1988; Roessler and Rosenberger, 1989) and the production of local protective immunoglobulins.

Although much has been done on the systemic antibody response to avian reovirus, untill the work described in this thesis, limited work has been reported on the role of local intestinal responses to infection (Watanabe et al., 1975). In most of the reports on local antibody production to infection in chickens, *in vivo* methods have been used. However, recently Zigtermann et al. (1993) described a technique whereby paired explanted samples of tissue are incubated at 4^0C and 37^0C. After incubation for 18 hours, an ELISA is used to compare amounts of antibody produced at the two temperatures. The assumption is that antibody detected at 37^0C but not at 4^0C would have been produced by the tissue. This method has been used in this experiment.

In the previous experiment, avian reovirus strain R2 was shown to replicate in all regions of the gut when a high inoculum (4.0 $\log_{10}$) was used. A virus dose close to this was therefore chosen for this experiment.

The purpose of this experiment was to support our findings in Chapter 4, that reovirus-specific antibodies are produced locally in the intestine and determine which part(s) of the intestine are involved.

8.5 Materials and methods

8.5.1 Chickens

Six 4-week old SPF chickens which had been kept in isolation and given food and water *ad libitum* were used.

8.5.2 Virus

Avian reovirus R2 already described in Chapter 3 was used.

8.5.3 Experimental design

Four of the chickens were inoculated with 0.3 ml of medium containing 3.5 $\log_{10}$ $TCID_{50}$ of reovirus R2 per 50µl. The other two were given medium without virus and kept as controls.

On day 14 pi, all the birds were humanely killed. From each bird, the intestines were carefully removed and each separate region of the gut was put in a petri dish containing pre-warmed medium (see Experiment 1). Intestinal organ cultures were harvested from each of the duodenum, jejunum, ileum, caecum and rectum and placed in test tubes as in Experiment 1 of this chapter.

Thus there were 10 test tubes each containing two pieces of each tissue from the infected birds and a similar number from the controls.

Selected intestinal samples from the control birds were taken for virus isolation to ascertain that they were negative for reovirus infection.

In order to assess local antibody production, tubes for each region were divided into two sets, whereby one was kept in an incubator at 37^0C and the other at 4^0C in a cold room. At both temperatures, cultures were kept on roller drums. Just before incubation, the medium from all the test tubes was collected and this was referred to as samples at time 0 (t=0), fresh medium was added and then incubation proceeded. After 18 hours (t=18) the medium was harvested from each tube. Samples from each gut tissue were pooled.

ELISAs for reovirus-specific IgA and IgG were performed on supernantant samples as already described in Chapter 3.

The increase in OD was calculated by subtraction of the OD at t=0 from the ones obtained at t=18 for the two temperatures. A reduction in OD at 4^0C was deemed to be indicative of local active secretion. A reduction greater than x3 has been considered 'significant' (Brito et al., 1993).

8.5.4 Immunoglobulin assays

IgA and IgG were assessed in samples by ELISA as already described in Chapter 3.

8.6 Results

8.6.1 Virus isolation

No reovirus was isolated from the control birds.

8.6.2 Reovirus-specific IgA secretion *in vitro*

Figures 6 and 7 show the results of the *in-vitro* IgA production at 37^0C and 4^0C in the intestinal explants of the control and infected groups respectively. The OD values of the controls (Figure 6) were low in both 37^0C and 4^0C explants compared to those of the infected groups, indicating lack of virus-specific IgA.

For the infected chicks (Figure 7), in the explants kept at 37^0C, the highest OD values were in the duodenum, ileum and jejunum, with lowest in the caecum and rectum. For those kept at 4^0C there were consistently low OD values, indicating lack of active IgA production. Results for the explants from the infected chicks (Figure 7) are described in detail below.

Duodenum

There was a four-fold difference in virus-specific IgA between the explants kept at 37^0C and those kept at 4^0C, indicating substantial local production.

Jejunum

Results were similar to those for duodenum except that the total amount of IgA was lower.

Ileum

Again a substantial production of virus-specific IgA was detected of a similar order to the duodenum.

Caecum and Rectum

Although IgA production was low at 37^0C, there was little or none when the explants were kept at 4^0C, indicating possibly very low levels of antibody production.

8.6.3 Reovirus-specific IgG secretion *in vitro*

Figures 8 and 9 show the results of assays for *in-vitro* virus-specific IgG antibody production at 37^0C and 4^0C. The low OD values for both 37^0C and 4^0C explants from control chicks (Figure 6) compared to those of the infected chicks (Figure 9), indicate that there was no virus-specific IgG in the control birds. The OD values of the explants kept at 4^0C were highest in the duodenum, rectum, caecum, jejunum and lowest in the ileum. Those kept at 37^0C were highest in the rectum, caecum, ileum and jejunum and lowest in the duodenum. Results for IgG assays (Figure 9) are detailed below.

Duodenum

After incubation for 18 hours, the amount of IgG released from the samples kept at 37^0C was about half of that released when the explants were kept at 4^0C. No local IgG was produced.

Jejunum

Substantial and similar amounts of IgG were detected in the jejunal explants kept at 37^0C and 4^0C. No local IgG antibody was produced.

Ileum

There was a reduction of more than two thirds in the IgG in the ileal explants kept at 4^0C when compared to that at 37^0C. This appeared to be the only site where virus-specific IgG was produced locally.

Caecum and Rectum

Both sets of explants kepts at 37^0C and 4^0C produced substantial amounts of IgG without any difference between them, indicating no local virus-specific IgG.

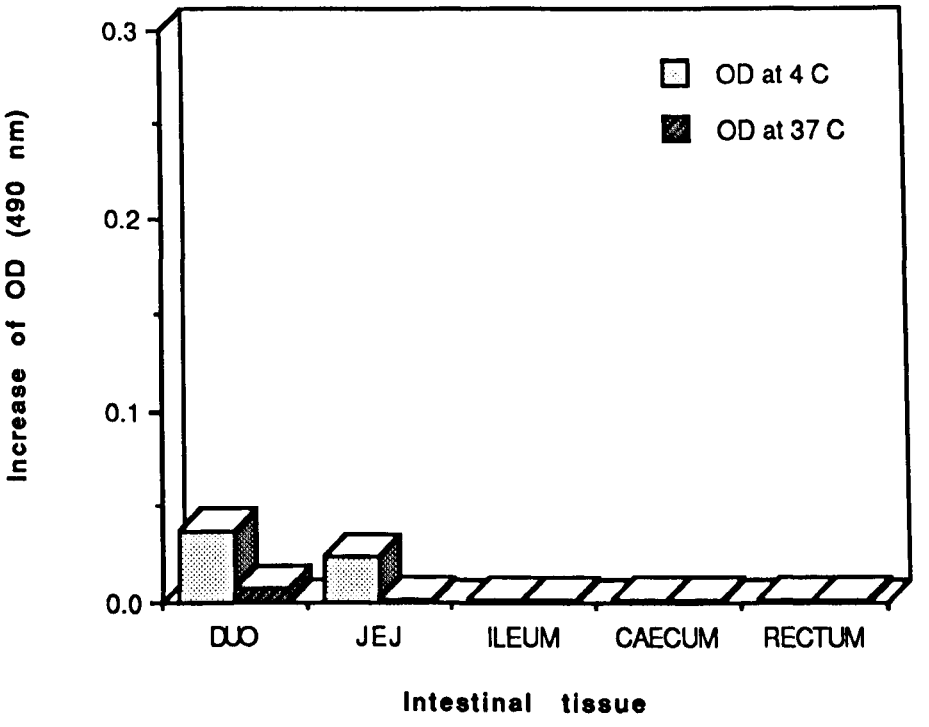

Figure 6. Local virus-specific IgA production in the supernatants of intestinal tissues from control chicks after *in vitro* incubation at 4^0C or 37^0C on day 14 pi.

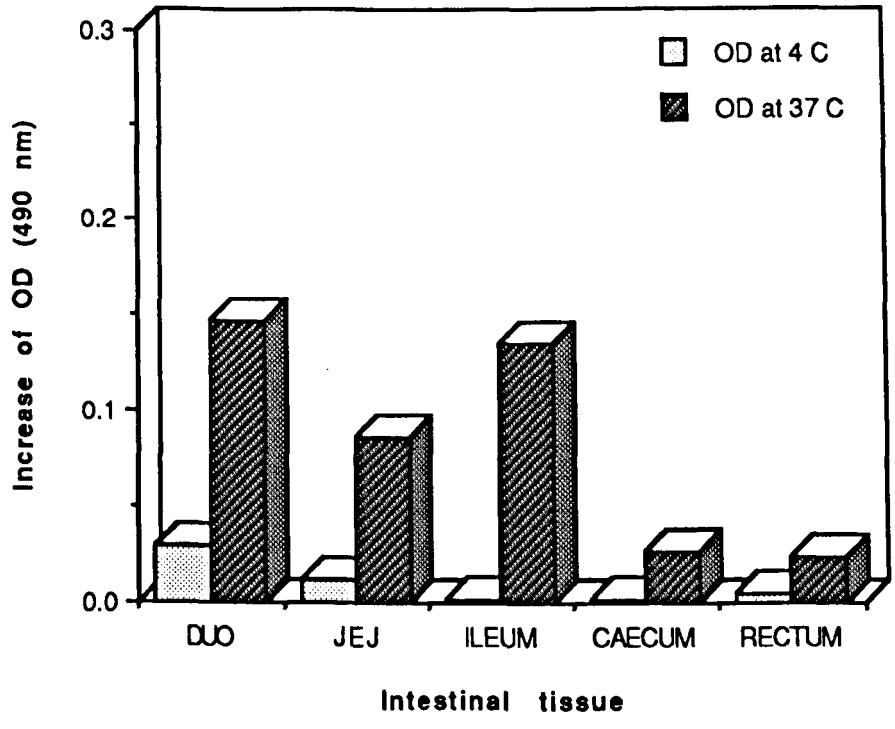

Figure 7. Local reovirus-specific IgA production in the supernatants of intestinal tissues from chicks infected orally when day-old after *in vitro* incubation at 4^0C or 37^0C on day 14 pi.

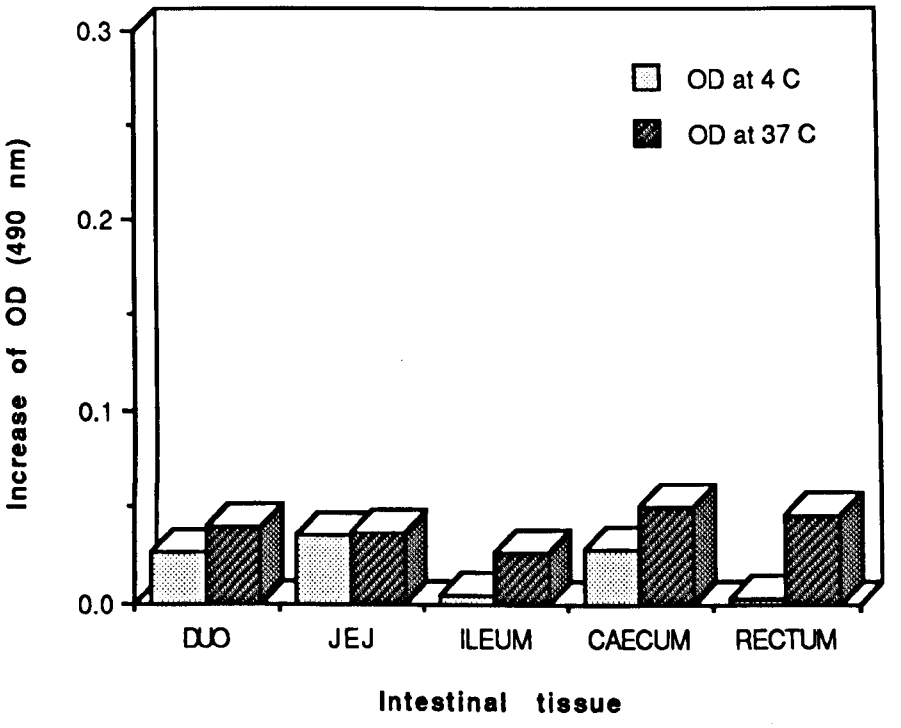

Figure 8. Local virus-specific IgG production in the supernatants of intestinal tissues from control chicks after *in vitro* incubation at 4^0C or 37^0C on day 14 pi.

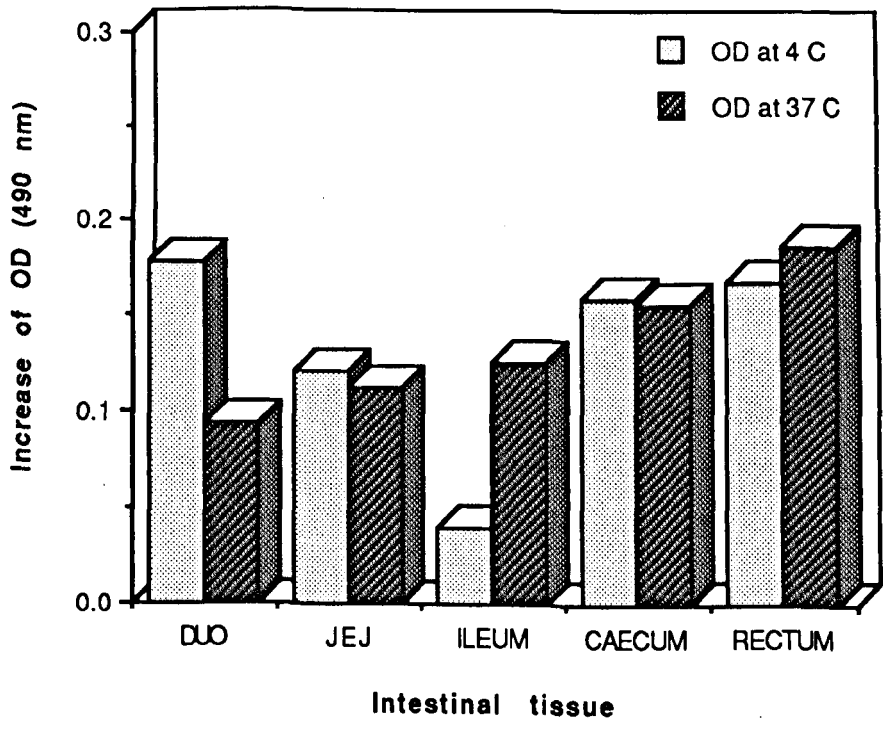

Figure 9. Local virus-specific IgG production in the supernatants of intestinal tissues from chicks infected orally when day-old after *in vitro* incubation at 4^0C or 37^0C on day 14 pi.

8.7 Discussion

The replication of avian reoviruses in enteric tissues is well documented. Because high titres and frequent isolations were obtained in the duodenum, jejunum and ileum (Kibenge et al., 1985; Jones et al., 1989), it was suggested that primary multiplication of virus occurs mainly in the duodenum and jejunum and viral titres in the posterior regions of the intestine to some extent reflected the amounts of virus shed from the anterior parts. Nevertheless, some studies have reported isolation of virus from the caecum and rectum (Menendez et al., 1975; Kibenge et al., 1985) (and see chapter 4).

Since the above studies used *in vivo* methods, it was not possible to quantitatively differentiate virus which may have originally replicated in the anterior part of the gut and later shed in the posterior part. Thus the first experiment in this study has used an *in vitro* method to further clarify reovirus replication in different tissues of the intestine.

The results showed that avian reoviruses are capable of replicating in all tissues of the intestine tested and this confirms the report by Kibenge et al., (1985) who infected day-old chicks orally with 4.8 $\log_{10}$ $TCID_{50}$ of virus and detected virus in the duodenum, ileum, caecal tonsils and rectum in increasing titres up to 5 days pi. However, the peak titres were lower in their experiment compared to ours, presumably because this one was an *in vitro* method.

The extent to which this replication may be influenced by the difference in virulence of the virus is not clear. For example, Rosenberger et al. (1989), found that after experimental infection, highly pathogenic isolates were distributed in liver, trachea and intestines and they persisted longer than less pathogenic isolates.

Furthermore, Mosos (1994) described differences in growth of isolates in the duodenum and suggested that some strains grew differently in different tissues.

Our results show an increasing virus replication pattern with graded virus inoculum from 2.0 to 4.0 $\log_{10}$ $TCID_{50}$ per 50µl virus titres used. The dose-related response in chicks after infection with avian reovirus is well documented. Wood and Thornton (1981) reported an increase in death rate and sickness with increase in dose of virus. Kataria et al. (1988) also recorded higher mortality (58%) in chicks after infection with a higher dose of virus than in those infected (12%) with a lower dose.

In a dose-respose experiment (Jones et al., 1994) using a trypsin-sensitive avian reovirus intranasally, reported similar results, whereby virus was isolated at 2 days pi from the jejunum following infection with reovirus doses of 3.0 $\log_{10}$ and above, but not lower. However, these results contrast with those of Kibenge et al. (1987), who reported that the infecting dose of strain R2 virus, ranging between 10^0 and 10^5 $TCID_{50}$/chick, had no marked effect on (1) the number of birds which shed virus via the cloaca each week, (2) the duration of shedding, (3) the antibody responses to reovirus, (4) the number of birds from which virus could be isolated from tendon tissue, or (5) the number with microscopic tenosynovitis at 5 weeks p.i.

Although older birds are relatively resistant to reovirus infection and virus is rarely isolated from the gut tissues beyond 3 weeks of age (Rosenberger et al., 1989; Roessler and Rosenberger, 1989), virus replicated well in the explants used here which were derived from 4 week-old birds. This indicates that age-related resistance to infection with reovirus could be caused by mechanisms which are extrinsic to these tissues e.g the immune response.

Although antigen-specific IgA has been reported in a number of infections of the chicken intestine (Orlan and Rose, 1972; Watanabe et al., 1975; Lee and Hanson, 1975; Jayawardane and Spradbrow, 1995; Parry et al., 1977; Myers and Schat, 1990), the methods of monitoring used have been mainly *in vivo* and it has not been possible to ascertain which region of the gut was involved in the secretion. In some cases, the gut contents were pooled or if not pooled could easily be mixed up. To measure local antibody production *in vitro* the method described by Zigterman et al. (1993) was used. A similar method had been used by Brito et al. (1993) whereby he indicated that salmonella-specific IgA were produced after oral infection of 5 week-old birds with *Salmonella typhimurium*. This method has advantages of avoiding the harsh environment of the gut and clearly indicates which regions of the gut secrete the immunoglobulin.

In the study of local IgA and IgG production (Experiment 2), 4 weeks of age chicks were used, because in a previous study (Chapter 4 Part 1) no virus-specific IgA could be detected in the intestinal contents of chicks infected when DO.

The results presented in here clearly indicate that there is local production of reovirus-specific IgA in the intestinal tissues of chicks infected with avian reovirus strain R2. The duodenum, jejunum and ileum showed the highest secretion of IgA in the anterior part of the gut. Virus-specific IgA production in the posterior part of the gut was limited, as shown by the low OD values in the caecum and rectum. Although these results are from explants of chicks infected orally when four week-old and sampled only on day 14 pi, they suggest that the anterior part of the gut is more active in local antibody production after reovirus infection.

With regard to virus-specific IgG, there were almost identical OD levels in the tissues incubated at 4^0C and at 37^0C for the jejunum, caecum and rectum.

This indicated that the IgG detected at both temperatures was not actively secreted during the incubation period, but was perhaps present as result of transudation from the circulation. Dhinakar Raj and Jones (1996) have recently described the transudation of infectious bronchitis-specific IgG from the systemic circulation into the duodenum between days 7 to 23 pi. The ileum appeared to be the only site among those tested which locally produced virus-specific IgG. Previously, however, by measuring virus-specific IgG in the intestinal contents, it was not possible to detect any IgG (Chapter 4). This discrepancy may confirm that IgG in the intestinal contents undergoes degradation.

An anomalous result was recorded in the duodenum, where there was a substantial reduction in virus-specific IgG when the explants were kept at 37^0C compared to 4^0C. Perhaps this could have been the result of enzymic degradation in this part of the gut, because it is in the duodenum where the pancreatic duct opens. Although the explants were washed, traces of enzyme could have remained and would have been more active at 37^0C than at 4^0C.

The protective role of IgG against systemic disease is well-established and IgG has been used as a measure of exposure to infection or vaccination in many disease conditions. However, little is known about the protective role of IgG in the mucosal tissues of the intestine. Attempts to detect virus-specific IgG in intestinal contents and bile have had little success and the levels so detected have been described as very low (Mocket, 1986; Myers and Schat, 1990). However, passive IgG has been shown to induce resistance to rotavirus infection in turkeys (Shawky et al., 1993) and systemic IgG has also been suggested to protect the gut against reovirus infection in mice (Barkon et al., 1996).

In conclusion, anterior regions of the chicken intestine are more active in secretion of virus-specific IgA than the posterior end. With regard to IgG secretion, the ileum was found to be the only active region. Thus, local IgA is likely to be actively involved in intestinal protection while IgG may act passively.

CHAPTER 9

PRELIMINARY STUDIES ON CHANGES IN TRYPSIN-SENSITIVITY OF AVIAN REOVIRUS STRAINS TR1 AND S1133 AFTER *IN VIVO* PASSAGE IN CHICKS

CONTENTS PAGE

CHAPTER 9

PRELIMINARY STUDIES ON CHANGES IN TRYPSIN-SENSITIVITY OF AVIAN REOVIRUS STRAINS TR1 AND S1133 AFTER *IN VIVO* PASSAGE IN CHICKS

9.1 Introduction

Mammalian reoviruses are classified into three serotypes, 1, 2 and 3 on the basis of serum neutralisation and haemagglutination inihibition tests (Rosen, 1960). Type 3 is sensitive to proteolytic enzymes trypsin and chymotrypsin while type 1 and 2 are resistant. Although all avian reoviruses had been considered to be trypsin-resistant (Kawamura et al., 1965; Petek et al., 1967; Jones et al. 1975), more recent studies have shown that some are trypsin-sensitive. Al-Afaleq and Jones (1991) described a trypsin-sensitive avian reovirus designated TR1, originally isolated from the hock joint of a turkey with arthritis. The infectivity of TR1 in CELi was reduced by about 3.0 $\log_{10}$ following treatment with 0.01% trypsin for 30 minutes, while that of a trypsin-resistant strain R2 was unaffected.

Futhermore, Drastini et al. (1994) investigated the trypsin and chymotrypsin sensitivity of 13 strains of avian reoviruses and found that 0.5% of chymotrypsin reduced the infectivity of all of them while 0.1% of trypsin affected 9/13 strains. Recent examination of 25 reovirus strains from various parts of the world demonstrated that 11 had sensitivity to trypsin similar to that of strain TR1 (Al-Mufarrej, 1993; Jones et al., 1996).

The trypsin-sensitivity of strain TR1 has been shown to affect its ability to replicate in the intestine and in turn, affect its spread to other target tissues such as the joint (Al-Afaleq and Jones, 1991;

Jones et al., 1994) and its egg transmission (Al-Mufarrej et al., 1996).

It has also been demonstrated that the trypsin sensitivity property of some avian reoviruses may not be stable. The segmented nature of the genome of reoviruses is said to be responsible for the genetic instability of these viruses (Wenske et al., 1985). Mosos (1994) examined several cloacal swabs of chicks infected orally when day-old with a trypsin-sensitive avian reovirus strain S1133 and found that the virus was capable of changing from a sensitive to a resistant form after one *in vivo* passage.

Since avian reoviruses are primarily pathogens which replicate in the gut, their spread is mainly oral-faecal. Both of these activities are substantially reduced by the effect of trypsin in the gut, so it is difficult to understand how these trypsin-sensitive strains survive in nature.

With increased interest in exploring induction of intestinal immunity as means of protection after vaccination with avian reoviruses, more knowledge on the role and mechanism of trypsin-sensitivity will be required, especially since the strain used most widely in vaccine development has been shown to be sensitive to the enzyme (Jones et al., 1996).

In Experiments 1 and 2, the effect of a single *in vivo* passage on the sensitivity of avian reovirus strains TR1 or S1133 respectively was investigated. In the third experiment, the effect of route of infection *in vivo* on the sensitivity is examined.

EXPERIMENT 1. CHANGES IN SENSITIVITY TO TRYPSIN OF AVIAN REOVIRUS STRAIN TR1 AFTER A SINGLE *IN VIVO* PASSAGE THROUGH TWO-WEEK OLD CHICKS INFECTED ORALLY.

9.2 Materials and methods

9.2.1 Chickens

Ten two-week old SPF chickens were used.

9.2.2 Virus

The trypsin-sensitive avian reovirus strain TR1, already described in Chapter 4 was used.

9.2.3 Experimental design

The ten 2 week old SPF chickens were put in individual cages where water and food was provided *ad libitum*. All the procedures of feeding and cleaning were carried out in such a manner that there was no cross-contamination between these birds.

Each of the birds was infected orally with 6.0 $\log_{10}$ $TCID_{50}$ of virus. Cloacal swabbing of each of the birds was done on alternate days until the termination of the experiment on day 14 pi.

At the end of the experiment, all birds were humanely killed. Tissues were taken from the intestine and tibiotarsal-tarsometatarsal (hock) joints for virus isolation, titration and trypsin sensitivity assay.

9.2.4 In vitro trypsin-sensitivity assays

Concentrations of trypsin (Gibco Ltd, Scotland) of 0.1% were prepared in phosphate-buffered saline (PBS), pH 7.2 in 0.9 ml

volumes. A 0.1 ml volume of each virus in growth medium M199 was added to a trypsin concentration of 0.1%. The same volume of virus was added to 0.9 ml PBS as untreated virus control. Each sample was mixed thoroughly and immediately placed in a 37^0C water bath. After 30 minutes, the samples were placed in iced water and at the same time 0.1 ml of each sample was put in 0.9 ml of growth medium containing 10% new-born calf serum to inactivate the enzyme.

9.2.5 Virus isolation and titration

Titrations were done on the following samples before and after trypsin treatment (see above): (a) the original inoculum given to the chickens, (b) virus recovered after cloacal swabbing, (c) virus isolated from hock joints or intestinal tissue at post mortem on day 14 pi. Isolations and titrations were done as already described in Chapter 3.

9.3 Results

9.3.1 Virus isolation and titration

Virus isolation results from cloacal swabs are shown in Table 1. Virus was isolated from 3 of 10 cloacal swabs of chicks on day 3 pi only. The virus was very low in quantity and could only be recovered after a third passage. No virus was recovered from the intestinal or the hock joint tissue on autopsy at 14 days pi.

In order to examine the trypsin sensitivity of the virus samples, the freshly isolated virus was passaged three times to raise the titre.

9.3.2 Trypsin sensitivity

In the virus which had been used as the inoculum, there was a 2.51 $\log_{10}$ reduction after exposure to trypsin (Table 2). However, after trypsin treatment, all the virus samples recovered from the cloacal swabs were found to have changed from trypsin-

sensitivity to resistance, with some actual enhancement. The change to resistance was between 0.33-1.05 $\log_{10}$.

Table 1. Virus isolation from cloacal swabs taken on alternate days from chicks infected orally when two-weeks old with strain TR1. (3) : isolated on third passage.

	Days pi			
Bird No	1	3	5	7-14
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	+(3)	-	-
6	-	+(3)	-	-
7	-	+(3)	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-

Table 2. Change in infectivity after trypsinization of the virus recovered from the cloacal swabs at day 3 pi from chicks infected orally when two-weeks old with strain TR1. Titres in $\log_{10}$ $TCID_{50}$ per 50µl

Bird No	Non-treated	Treated	Difference
5	4.63	5.38	+0.75
6	4.63	5.68	+1.05
7	4.50	4.83	+0.33
Inoculum	4.83	2.32	-2.51

EXPERIMENT 2. CHANGES IN SENSITIVITY TO TRYPSIN OF AVIAN REOVIRUS STRAIN S1133 AFTER A SINGLE *IN VIVO* PASSAGE THROUGH TWO-WEEK OLD CHICKS INFECTED ORALLY.

9.4 Introduction

A number of the current avian reovirus vaccines are derivatives of strain S1133. Recent studies (Jones et al., 1996) have shown that this strain is trypsin-sensitive. In Chapter 4 it was shown that trypsin-sensitivity is likely to affect the local immune response in the gut of such strains. Change in trypsin-sensitivity of S1133 may be an important parameter to consider when evaluating the local intestinal immune responses during vaccine development. In this experiment the virulent form of strain S1133 was used.

9.5 Materials and methods

9.5.1 Chickens

These were two-week-old SPF chicks as already described in Experiment 1 of this chapter.

9.5.2 Virus

Strain S1133 was originally isolated from viral arthritis (van der Heide and Kalbac, 1975) and has been the main source of many commercial reovirus vaccines. The virulent form was used in this experiment. The virus has been shown to be sensitive to trypsin (Mufarrej, 1993; Jones et al., 1996). It was used at a titre of 6.0 $\log_{10}$ per 50µl.

9.5.3 Experimental design and *in vitro* sensitivity assays

These were as already described in Experiment 1 of this Chapter.

In this experiment virus was titrated directly and then treated with trypsin, ie not passaged to increase titre. This was done to obviate the possibility that *in vitro* passage affects sensitivity.

9.6 Results

9.6.1 Virus isolation and titration

Virus isolation results are shown in Tables 3 and 4.
Virus was isolated from the cloacal swabs of 8/10 birds on day 1, 8/10 on day 3 and 6/10 on day 5 pi. None was recovered from swabs taken on days 7-14 pi.

Five of 10 joint tissues collected at day 14 pi were positive for virus (Table 4) but all intestinal tissues were negative.

9.6.2 Trypsin sensitivity

After trypsin treatment (Table 5), virus recovered from the cloacal swabs on day 1 pi, had a reduction in titre ranging from 0.82 to 1.31 $\log_{10}$ while the original inoculum had a reduction of 2.88 $\log_{10}$. By day 5 pi, of the six samples isolated only one still showed sensitivity, and four showed enhancement of titre (range +0.21 to +1.12 $\log_{10}$).

Although the virus samples recovered from the joints on day 14 pi (Table 6) were still sensitive to trypsin (range -0.15 to -1.94 $\log_{10}$), the sensitivity was less than that of the original inoculum.

Table 3. Virus isolation from cloacal swabs of chicks infected orally when two-weeks old with reovirus strain S1133.

Bird No	Days pi 1	3	5	7-14
1	-	+	+	-
2	+	+	+	-
3	+	+	+	-
4	+	+	-	-
5	+	+	+	-
6	+	+	+	-
7	+	+	-	-
8	+	+	+	-
9	+	-	-	-
10	-	-	-	-

Table 4. Virus isolation results on day 14 pi, from the hock joints and intestinal tissue of chicks infected orally when two-weeks old with reovirus strain S1133.

Bird No	Joint	Intestine
1	+	-
2	+	-
3	-	-
4	+	-
5	+	-
6	-	-
7	-	-
8	+	-
9	-	-
10	-	-

Table 5. Alterations in titres after *in vitro* trypsin treatment of S1133, re-isolated from the cloacal swabs of chicks infected orally when two-weeks-old. -: not done; -ve: no virus isolated.
Inocm: Inoculum.

Bird No	Day 1 pi			Day 5 pi		
	Non-treated	Treated	Difference	Non-Treated	Treated	Difference
1	4.63	3.5	-1.13	1.83	1.32	-0.51
2	3.32	2.5	-0.82	2.63	2.83	+0.2
3	5.17	4.63	-0.54	1.38	2.5	+1.12
4	4.0	3.17	-0.83	-ve	-	-
5	3.83	2.63	-1.2	2.47	2.68	+0.21
6	4.63	2.83	-1.8	3.17	2.17	+1.0
7	-ve	-	-	-ve	-	-
8	2.63	1.32	-1.31	2.17	2.17	0.0
9	2.17	1.32	-0.85	-ve	-	-
10	-ve	-	-	-ve	-	-
Inocm.	3.38	0.5	-2.88			

Table 6. The effect of trypsin on infectivity of reovirus strain S1133 after re-isolation from the hock joints at day 14 pi of chicks infected orally when two-weeks-old

Bird No	Un-treated	Treated	Difference
1	1.32	1.17	-0.15
2	3.32	1.38	-1.94
3	-ve	-	-
4	2.17	1.17	-1.00
5	1.83	0.5	-1.33
6	-ve	-	-
7	-ve	-	-
8	2.5	1.32	-1.18
9	-ve	-	-
10	-ve	-	-
Inoculum	3.38	0.5	-2.88

EXPERIMENT 3. THE EFFECT OF ROUTE OF INFECTION ON THE KINETICS OF TRYPSIN-SENSITIVITY TO STRAIN TR1 IN CHICKS INFECTED AT DAY-OLD

In Experiments 1 and 2, it was shown that the trypsin-sensitivity of strains TR1 and S1133 changed after a single *in vivo* passage through chicks when given orally. The purpose of this experiment was to investigate whether administration of TR1 via the footpad (FP) route, which is distant from the source of trypsin (gut), has any effect on the kinetics of trypsin-sensitivity.

9.7 Materials and methods

9.7.1 Chickens

Day-old SPF chicks were used and kept as already described in Experiments 1 and 2.

9.7.2 Virus

Trypsin-sensitive avian reovirus TR1 was used.

9.7.3 Experimental design

Thirty day-old chicks were divided into two groups of 15 each. One group was infected by the footpad route with 0.2 ml per chick (0.1 ml each foot) of medium M199 containing 6.17 $\log_{10}$ $TCID_{50}$ per 50µl of TR1. The second group was infected with the same dose of virus orally.

On days 2, 7 and 14 pi, five birds from each group were sacrificed and sampled. Representative pieces of the intestine (duodenum, jejunum and ileum) and both hock joint tissues were collected for virus isolation and subsquently trypsin-treatment as already described in Experiment 1.

In many cases, the virus isolated proved too low to be titrated, and hence all samples were passaged three times to increase their titre before trypsin-treatment.

The mean titres from the inoculation groups were compared for statistical significance using the students t-test ($p<0.05$).

9.8 Results

9.8.1 Virus isolations and trypsin treatment

Results of the the trypsinization of virus isolated from the joint and intestinal tissues of chicks infected orally or by footpad when day-old with reovirus strain TR1 are shown in Table 6.

Virus was recovered from the hock joints of all chicks infected orally or by footpad on days 2, 7 and 14 pi., but intestinal tissues were positive on days 2 and 7 pi only.

After trypsin-treatment, the virus used as inoculum had its titre reduced by 2.33 $\log_{10}$.

After trypsin-treatment there was no significant difference ($p<0.05$) in reduction or change in sensitivity to trypsin between the virus recovered from the joints, on days 2, 7 and 14 pi. whichever route was used, although the virus recovered on day 14 had a much reduced titre than that recovered on days 2 and 7 pi.

Overall, the virus recovered from the joints (range -0.84 to -2.01 $\log_{10}$) had a less reduced sensitivity to trypsin than that recovered from the intestine (range -0.91 to -1.51 $\log_{10}$) (Table 6)

There was no significant difference in reduction in trypsin sensitivity between virus recovered from the intestine on different days pi. with either route.

Table 6. Mean (n=5) titres (SD) of virus before or after trypsin-treatment with 0.1% trypsin from joint or intestine of chicks infected by footpad or orally when day-old with a trypsin-sensitive reovirus strain TR1. FP=footpad; -ve= no virus isolated.

Days pi	Tissue	Non-treated	Treated	Difference
2	Joint(FP)	5.63±0.14	3.75±0.26	-1.88±0.32
	Gut(FP)	5.54±0.53	4.16±0.33	-1.39±0.25
	Joint(Oral)	5.78±0.16	4.07±0.15	-1.72±0.09
	Gut (Oral)	5.37±0.36	3.86±0.31	-1.51±0.61
7	Joint(FP)	6.38±0.50	4.37±0.51	-2.01±0.24
	Gut(FP)	5.94±0.44	4.70±0.42	-1.24±0.23
	Joint(Oral)	5.50±0.28	3.84±0.56	-1.67±0.45
	Gut(Oral)	4.95±0.60	4.04±0.51	-0.91±0.79
14	Joint(FP)	5.40±1.30	3.53±1.09	-1.60±0.65
	Gut(FP)	-ve		
	Joint(Oral)	5.50±0.56	4.67±0.43	-0.84±0.51
	Gut(Oral)	-ve		
	Inoculum	5.5	3.17	-2.33

9.9 Discussion

The results obtained in the first experiment show that negligible virus was recovered from the cloacal swabs because most of the virus used as the inoculum was inactivated by trypsin and perhaps other related enzymes (Jones et al., 1996). The age of the chicks used in this experiment is also likely to have contributed to the low recovery of TR1 as well (see Chapter 4).

The virus recovered on day 3 pi, was found to be already trypsin-resistant. The short period between the time of infection and recovery of the trypsin-resistant virus is a surprise. These results suggest that there could have been a trypsin-resistant subpopulation in the inoculum, which survived the effect of trypsin. However, when this kind of result was obtained by Mosos (1994), he examined numerous plaques of TR1 inoculum but failed to isolate a resistant subpopulation.

Another possible mechanism by which such a change could occur is by mutation. Point mutations have been suggested as mechanisms by which vaccine strain S1133 could have changed to become part of the circulating poultry virus population (Rekik and Silim, 1992). Besides, it is well known that RNA viruses have high mutation rates, which provide a mechanism for rapid genomic variation (Holland, 1984). The short time this could have taken to occur makes this hypothesis difficult to believe. However, reoviruses have been isolated from the liver as soon as six hours after oral infection (Jones et al, 1989), and they suggested that this was due to a rapid transport of virus into the bloodstream. Studies with mammalian reoviruses have shown that the replication cycle of reoviruses could be as short as 12 hours after infection, and at this time most of the proteins synthesized in infected cells are viral proteins (Tyler and Fields, 1990).

In the second experiment, another trypsin-sensitive reovirus S1133, a virus which forms the basis of many reovirus vaccines and recently found to be trypsin-sensitive (Jones et al., 1996), was

used instead of TR1. This virus appeared to be more virulent than TR1 and was readily recovered from more chicks and for a much longer time than TR1 in Experiment 1. It might have been argued that S1133 is less sensitive to intestinal trypsin and other enzymes than TR1, but this is not likely to be correct because the inoculum of S1133 was shown to be more sensitive to trypsin than TR1. Possibly, the difference in their pathogenesis could be due to host differences, since TR1 is an isolate from turkeys (Al-Afaleq and Jones, 1989) while S1133 is more adapted to chickens.

The virus recovered on day 1 pi from the cloacal swabs of chicks infected orally with S1133 had a reduction in sensitivity ranging from -0.82 to -1.31 $\log_{10}$ compared to that of the inoculum which was -2.88 $\log_{10}$.
Such a difference indicates that even by day 1 pi, some changes in sensitivity had already started. This was conclusively demonstrated by the results of trypsin-treatment of the virus recovered on day 5 pi. Most of that virus had changed to trypsin-resistance by then. The results in this experiment in addition to those in the first study show that changes in sensitivity occur very fast.

Although the virus recovered from the hock joints on day 14 pi had lesser reduction in sensitivity (range -0.15 to -1.94 $\log_{10}$), compared to the inoculum, there was no trypsin-resistant virus detected. This indicates that when virus gets to sites where there is no enzymic activity (e.g hock joints), the pressure to change is reduced and it maintains its original state. Presumably this means that the virus reached the hock joints soon after infection, avoiding enzyme activity in the gut.

The third experiment was meant to examine whether the route of infection has any effect on the changes in the sensitivity of these trypsin-sensitive reoviruses. The chicks used in this experiment were DO when infected with TR1 and this may explain the greater frequency of virus isolation than in Experiment 1 where the chicks were two weeks old. In Experiment 3 the dramatic change from sensitivity to resistance seen in Experiments 1 and 2 did not

occur. This too may have been due to the young age of the chicks in that there may have been relatively less trypsin for virus inactivation than in the older birds. In spite of the above, results showed that there is no effect of route of infection to changes in sensitivity, other than the site where the enzymes are present. Thus, the virus isolated in the hock joints had identical trypsin sensitivity irrespective of route of inoculation.

All viruses isolated from the intestinal tissues of chicks in experiment 3, even by day 7 pi, were still sensitive to trypsin, while those isolated from the cloacal swabs in experiments 1 and 2 had already changed to resistant ones by days 3 and 5 pi. If this is not due to the age difference, then it is possible that the virus within the gut tissue may be protected from the enzymes just like that in the joints.

It can be concluded that changes due to enzymic pressure to the trypsin-sensitive reoviruses occur very rapidly after infection. Although viraemia is known to occur (Kibenge et al., 1985; Jones et al., 1989) soon after infection, virus tends to be localised in some sites thereby maintaining their trypsin-sensitivity nature. It may be wothwhile to examine the immune response induced by viruses which have undergone such changes. However if one considers, vaccinal derivatives of S1133, (i) they would be less virulent (ii) the doses would be so low and less likely to survive the trypsin in the gut.

CHAPTER 10

GENERAL DISCUSSION AND FUTURE WORK

This chapter highlights the most important deductions from the experiments described in this thesis and outlines the direction of future work. Briefly, the thesis examined the mechanisms of systemic and intestinal antibody responses of the chicken to avian reoviruses and attempted to establish how they may be affected by factors such as (a) the age at infection (b) route of inoculation (c) nature of the infecting of virus (i.e., whether resistant or sensitive to trypsin) and (d) immunosuppression of the B-cell system.

Many studies have examined the pathogenesis of reovirus-induced tenosynovitis but little attention has been paid to the mechanisms of the immune responses involved. Several have reported on the production of systemic antibody responses after reovirus infection, and these have been used to monitor efficacy of vaccinations but none has described local immune responses.

For satisfactory control of avian reovirus infection in chickens, it is necessary to understand the following complexity of events which are highly relevant: (a) Egg transmission of virus occurs at a low rate but most chicks become infected following horizontal spread soon after hatch. Birds become resistant to development of disease with age. This makes challenge studies in older birds of little value. (b) Although natural infection is mostly via the oral-faecal route, and virus replicates in the gut to high titre, there are no pathological signs in the intestines; instead, the important damage is inflicted further away, in the hock joints. Thus the intention of vaccination should be to prevent virus establishing in the joints at an early age and causing tenosynovitis, so the ideal approach is to

first stop the replication of virus in the gut. Vaccination of breeders with killed vaccines supplies the chicks with maternal antibodies, but they will not prevent enteric replication of challenge virus (Nwajei and Jones, 1985). Thus early vaccination of chicks with live vaccine should be more efficacious.

The first experimental chapter (4) explored the effects of age, route of inoculation and strain of reovirus in relation to trypsin sensitivity on virus-specific systemic and intestinal antibody production after infection. Virus-specific intestinal IgA was detectable only in chicks infected when one week-old and older and the oral route of inoculation was more effective than the subcutaneous. The trypsin-sensitive strain of reovirus did not replicate in the gut at the dose used and consequently, no IgA was produced. With regard to IgG, both trypsin-resistant and-sensitive strains induced systemic responses but with the subcutaneous route being superior. This work showed that with regard to enteric IgA production, day-old chicks could not produce it but 7-day old chicks could.

However, although unable to elicit IgA in the gut after day old infection, such chicks were able to produce this immunoglobulin in tears, as shown in in Chapter 6. This later work showed that lacrimal antibody could be important and that the eye-drop route of inoculation appeared to be superior to the conventional oral or subcutaneous routes. The appearance of lacrimal IgA and neutralising antibody in day-old infected chicks, which coincided with the cessation of virus replication in the HG and the fact that some of the virus given by eye-drop is swallowed into the gut, supports this. Coarse spray application of vaccines which has been recently introduced, could allow eyedrop and oral exposure to vaccines.

It should be noted that this work was done in SPF light-breed chicks. Similar studies should be done in broiler-type chicks which are more susceptible to reoviruses, and in chicks with maternal antibodies, to see how they may influence active antibody production.

How the presence of intestinal virus-specific IgA relates to protection against oral challenge will need further examination. While tears can be easily tested for virus neutralising activity, intestinal exudates, containing enzymes, bile salts etc., were toxic for tissue culture cells. In further studies, this could be obviated by purification of the IgA perhaps by precipitation and dialysis. A simple test for coproantibodies (although difficult to devise) could be a good indicator of intestinal protection and might be superior to the foodpad challenge method adapted as the standard in the USA.

More work is required to establish the balance between how early to vaccinate *vis a vis* the time when chicks are immunologically mature enough to produce intestinal immunity. Thus rigorous bio-security control measurses will remain essential to protect day-old chicks against field challenge

The work in this thesis has involved investigation of antibodies only. The role of non-antibody factors in protection against reovirus challenge after vaccination need to be examined. Evidence suggests that that cell-mediated immune responses, especially natural killer cells are involved in protection against coccidia (Lillehoj, 1991) and avian rotavirus (Schat and Myers, 1991). The use of T-cell immunosuppressor drugs such as cyclosporin and monoclonal antibodies for T-cell markers and T-cell receptors (TCR1 and TCR2) and improvements in methods of isolation of intra-epithelial leucocytes (Hoggenmueller, et al., 1993) may reveal the cell mediated mechanisms involved in intestinal protection against reovirus infection.

In reovirus infection of chickens, the subject of immunosuppression has been addressed in two different ways, depending on the hypothesis pursued. On the one hand, reovirus has been suggested to be immunosuppressive *per se* (Sharma, et al., 1994) and on the other, the effect of B-cell immunosuppression on chicks vaccinated against reovirus has

been reported upon (Kibenge, et al., 1987; Moradian, et al., 1990). Since the subject of this thesis was protection by vaccination, we looked at the effect of B-cell suppression on the intestinal and systemic immune response.

The increased persistence of reovirus in B-cell immunocompromised chicks reported by Kibenge et al. (1987) and Moraden et al. (1990), was confirmed in Chapter 5 and in addition, it was shown that the effect of B-cell suppression affects not only the systemic but also the local antibody in the gut. The effect of Bx on the intestinal IgA is likely to have been the reason for the greater persistence of virus, although the correlation of intestinal IgA with protection is not clear-cut. Virus-specific IgA was completely inhibited, while the appearance of IgG in serum was only delayed and reduced. If this relative suppression in class-specific antibody was to occur in nature, then immune status of the flock, is a factor to closely monitor, if the aim is to attain intestinal protection.

It is not known what effect B-cell suppression could have when adult birds are vaccinated with the intention of inducing maternal antibody. Since vaccination of breeders is still practiced and *in ovo* vaccination is currently under trials, more studies are required to establish how these vaccination strategies are affected by B-cell suppression Unfortunately, there is no simple accurate measure of B-cell suppression that can be used by the producer.

Several antigenic types of avian reovirus are known to exist and incomplete cross-protection has been demonstrated between a number of reovirus strains (Rau et al., 1980; Takase et al., 1996). It is likely to be a major reasons why vaccinations against reovirus infection are not always successful. Chapter 7 showed that the trypsin-sensitive strain TR1 was not protective against challenge by the related trypsin-resistant one (R2), although antibodies were produced by TR1 in sera and tears. Protection afforded by immunisation with R2 to challenge with the same virus was 100%, but strain TR1 did not protect against challenge with R2. This would again suggest that a virus like R2 would be a

better vaccine candidate than one which does not replicate in the gut and it strengths the hypothesis that local IgA in the gut is vital in protection against enteric challenge in older chicks.

If R2 were to be used in modified form as a vaccine, more studies would be essential to establish whether it is protective against infection with heterologous challenge strains.

Chapter 4 had already shown that older chicks are able to produce intestinal IgA after infection with a trypsin-sensitive strain reovirus R2 and the *in vitro* work in Chapter 8 added to this information by showing that active secretion of IgA occurs in the anterior part of the gut. Active secretion of IgA requires that virus should be present and replicating with these cells (Shaw and Hempson, 1996). Thus strain TR1 which does not readily replicate in the gut is likely to be a poor vaccine candidate. Presumably S1133-based vaccines behave similarly to TR1. In future it may be necessary to test vaccine virus candidates for trypsin-sensitivity.

Recent evidence suggests that a number of avian reoviruses are trypsin-sensitive and hence they replicate poorly in the gut. However, some of the sensitive strains have been reported to change to a resistant state after single passage *in vivo* (Mosos, 1994; Jones et al., 1996). Preliminary studies in Chapter 9 show that change from sensitivity to resistance is mainly a factor of the enzymic pressure in the gut and it was not affected by the route of inoculation. The change to resistance has also been shown to occur within a surprisingly short time after infection. It has not been tested whether after the change, such strains are able to replicate and induce intestinal IgA in the gut, but it seems likely. However, for the change to occur, it requires the use of very high virus doses, which would be higher than given in a vaccine. Thus it seems unlikely that a trypsin-sensitive vaccine would switch this way after normal application.

It is not known whether trypsin-resistant strains are able to revert to sensitive ones under certain unknown conditions. Even

the mechanism by which these changes occur, are yet to be determined. This will require an identification of the surface protein(s) affected by the enzyme and, in turn, recognition of the gene(s) responsible. Presumably, in view of the rapidity of the switch, a single gene is involved. It will be important to know if the switch is reversible, although the practical evidence suggests that it is not.

In all these experiments the fact that a trypsin-sensitive strain TR1 does not replicate (at the dose used or at a vaccine dose) and does not induce intestinal IgA response has remained valid.
Lack of effective practical vaccination regimes suitable for stimulation of intestinal mucosal IgA antibody, failure to elicit substantial mucosal responses using inappropriate antigens, may direct future activities towards alternative strains of reovirus (R2) and possibly the use molecular biology to establish viral components which induce better immunity in the gut.

Thus in terms of practical benefits of a vaccination strategy, two conclusions emerge. (i) Considering nature of virus, with the aim of protecting the gut by preventing virus replication, a vaccine derived from a trypsin-resistant strain, given orally, within one week of hatch, could be superior.
(ii) As for the route, with the aim of protecting the chick soon after hatch, administration by eye-drop or an improved coarse-spray method would be ideal.

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APPENDIX-A

Virus cultivation

Antibiotic solutions

Materials:

Crystapen (Glaxo Lab. Ltd., Greenford, England) (1,000,000 iu benzyl penicilin sodium BP)	600.0 mg
Streptomycin sulphate BP (Evans Medical Ltd., Greenford, England)	1.0 g
Sterile distilled water	40.0 ml

Method:

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

Fungizone (Amphotericin B) (E.R. Squibb & Sons, Inc., Princeton, New Jersey, USA)	50.0 ml
Sterile distilled water	50.0 ml

Method:

Fungizone was dissolved in sterile distilled water, divided into 2 ml aliquots and stored at $-20^{0}C$.

Dulbecco's phosphate buffer saline-A (PBS-A)
(i) Stock PBS-A solution

Materials:

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

Method:

All 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.

(ii) Working PBS solution
80 ml of stock PBS-A solution were diluted in 800 ml of sterile distilled water. 4 ml antibiotic solution was added. Working PBS was stored at 4^0C.

This buffer was used for cell culture and other purposes requiring sterile PBS.

Trypsin solution (0.05%)
Materials:

2.5% Typsin (1:250) (Difco Ltd., Paisley, Scotland)	10 ml
PBS-A	500 ml

Method:
The 2.5% trypsin solution was divided into 10 ml aliquots and stored at -20^{0}C. Immediately before use, one aliquot was thawed and diluted in 500 ml pre-warmed PBS-A. This was used for disaggregation of cells during the cell culture preparation.

The same stock of trypsin was used to prepare the various concentrations during the reovirus-trypsin sensitivity assays.

CELi cell culture medium M199
Materials:

Sterile distilled water	625 ml
M199 medium (10x,with L-glutamin(Gibco Labs)	85 ml
Tryptose phosphate broth	100 ml
7.5% Sodium bicarbonate (Gibco)	10 ml
1M HEPES buffer (Gibco)	14 ml
Antibiotic solution	4 ml
Fungizone solution	2 ml

Method:

The ingredients were added together and mixed gently. The pH was adjusted to between 7.2-7.4. Just before use, 10% or 5% of newborn calf serum was added to the medium to make growth or maintainance medium respectively.

Intestine organ culture

Intestine organ culture medium (MEM)

This medium was used for the study of virus growth in intestine organ culture.

Materials:

Distilled water	850 ml
MEM Eagles x10 (Gibco)	100 ml
7.5% sodium bicarbonate	30 ml
Gentamycin (10 mg/ml)	5.0 ml
Antibiotic solution	5.0 ml

Method:

Ingredients were added together and mixed gently. The pH was adjusted to between 7.2-7.4 10% or 5% foetal calf serum was added to the medium to make growth or maintainance medium respectively just before use.

ELISA

Coating buffer CBC 0.05M, pH 9.6

Sodium carbonate	1.50 g
Sodium bicarbonate	2.93 g

Method:

The buffer was made up to 1 litre and stored for not more than a week at -4^{0}C.

Phosphate citrate buffer

0.1M Citric acid	243 ml

0.2M Na_2HPO_4 added until pH 5.0

This was made up to 1 litre with distilled water

Substrate

0.4g ortho-phenylenediamine(OPD) was dissolved in phosphate citrate buffer, divided into 10ml aliquots and stored at -20^{0}C. Prior to use, 10 µl of 30% hydrogen peroxide were added to 10ml of thawed substrate. The solution was kept in the dark because its photosensitive.

Stock ELISA washing solution

$NaCl_2$	292.2 g
Na_2HPO_4	5.35 g
$NaH_2PO_42H_2O$	1.95 g
Tween 80	2.5 ml

Method:

These ingredients were mixed and made up to 5 litres.

Stopping solution

2.5M sulphuric acid (245g/litre or 134 ml/litre).

Plate washer

A titertek Handiwash 110 (ICN, High Wycombe, Bucks,England), eight channel washer was used at all washing stages.

Plate reader

Plates were read on a Dynatech (Billingshurst, Sussex) MR 700 microplate reader. This was set up to read at 490 nm (test filter 3) with a reference reading at 630nm (reference filter 5)

APPENDIX B

List of presentations

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

Mukiibi-Muka, G. and Jones, R.C. (1996). The response of the chicken intestine to reovirus infection: preliminary observations. In: International symposium on adenovirus and reovirus infections in poultry. (Eds by E.F. Kaleta and U. Heffels-Redmann) Rauischholzhausen, Germany, 24-27 June 1996. pp 245-250.

Mukiibi-Muka, G. and Jones, R.C. (1997). Stimulation of local immune responses in chicken by avian reoviruses: effects of route, age and virus. Paper presented to The British Veterinary Poultry Association, Spring Meeting, Harrogate, 8-10 April, 1997.Q