T-Cell Mediated Immunosuppression And Its Impact On Avian Infectious Bronchitis Virus Persistence In Chickens

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Introduction

Although infectious bronchitis (IB) is regarded as an acute respiratory disease of chicken, caused by Infectious Bronchitis Virus (IBV), long-term persistent infection with IBV in experimentally infected tissues such as kidney, caecal tonsil and rectum has been reported by Cook (1968) and Alexander *et al* (1978). A similar persistence of infection has been observed in fungal infections, where immune modulation, particularly in T-cell responses, plays a critical role in pathogen clearance or persistence (Pathakumari et al., 2022). El Houadfi *et al* (1986) also reported long-term persistence infection with IBV strain G (IBVG) in intestine of the chicken but not in respiratory tract. Day old chicken infected with IBVG did not excreted virus in their faeces until before 35-day post infection. However, re-excretion occurred when chicken reached their sexual maturity (Jones and Ambali 1987).

Treatment of chickens with oestrogen and progesterone after infection with IBV could not induced viral re-excretion (Ambali and Jones, 1990) but it did occur after T-cell suppression by cyclosporin (CSA) (Bhattacharjee *et al.*, 1995 and Dhinakar Raj and Jones, 1997). This drug (CSA) has been extensively used to study the importance of cellular immunity in a variety of infectious diseases affecting avian species such as turkey haemorrhagic enteritis (Suresh and Sharma 1995), marble spleen disease virus of pheasants (Fitzgerald *et al.*, 1995), reovirus (Hill *et al* 1989), and avian infectious bronchitis virus (Bhattacharjee *et al.*,1994; Dhinakar Raj and Jones 1997) CSA has been used as a means of inhibiting the cell-mediated immune response in order to determine the role of T-cells in protective responses to infectious pathogens of chickens (Fitzgerald *et al* 1996; Hill *et al* 1989; Isobe *et al*., 2000; Lillehoj *et al*., 1987). CSA selectively suppressed T-lymphocyte activity and cell-mediated immunity (Larsson, 1980; hill *et al* 1989; Ziprin *et al* 1989). As a consequence, all cell-mediated immune responses driven by cytokines, especially IL-2-dependent functions, which include T-helper activities, cytotoxicity, natural killer cell activity and antibody-dependent cell cytotoxicity, would be deleted after CSA treatment (Schreiber and Crabtree 1992).

The aim of this study was to repeat similar work done in this laboratory (Bhattacharjee *et al.*, 1994; Dhinakar Raj and Jones, 1997), whereby CSA was used to induce re-excretion of IBV after the acute phase of the infection had finished, to attempt to confirm that the kidney is the site of persistence, and to examine re-activated virus for evidence of mutation in the hypervariable region of the S1 spike gene. This was a short – term experiment which might suggest whether a longer-term infection would result in the generation of a genotypically different virus or viruses.

Materials and methods

1.Chickens

SPF eggs of white leghorn (WLH) chickens were obtained from a commercial source. They were hatched in our laboratory and the chickens maintained in complete isolation. They were fed and watered *ad-libitu*

2.Viruses

Chicks were inoculated by a combination of intranasally and intraocular application of 100□l of allantois fluid containing 4.9 $\log_{10}$ CID_{50} of IBV/ml of the Massachusetts M41strain.

3. Experimental designs

Fifty-six one-day old SPF chicks were infected with IBV strain M41. Following infection, samples of trachea, lung, kidney, caecal tonsil, brain, thymus, testes (from males) and duodenum were collected on day 3, 7, 10, 14, 21, 28 and 35 pi for VI in TOC and RT-PCR. Each time, five infected birds and three controls were examined. Twenty-nine chicks were kept as controls. After the birds had recovered fully from the acute phase of the disease, at 5 weeks of age, a course of intramuscular CSA injections (see below) was given, starting from day 34 pi and then, every three days until day 49, in attempt to induce re-excretion of virus.

Samples of trachea, lung, kidney, caecal tonsil, bursa, brain, thymus, and duodenum were collected 12, 15, 18 and 21 days post the onset of CSA treatment from three treated birds and two controls for examination by VI and PCR. Effectively, these were days 46, 49, 52 and 55 days of age.

4-Chicken embryo tracheal organ culture (TOC)

These were prepared and used for virus isolation and titration as described by Cook et al. (1976), The method for preparing TOC was as described by Cook et al. (1976). Briefly, embryos were removed from fertile 19 or 20-day-old SPF eggs and tracheas were collected from them after decapitation. They were freed from excess fat and other tissues and placed in a Petri dish contain warm (37oC) TOC medium Tracheas were cut in to 0.6mm thick rings using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd, Gomshall, Surrey, UK) and each ring was placed in a sterile glass tube (Nunc Life Technologies, Paisley, Scotland) containing 0.7ml of TOC medium. Tubes were replaced in a rotating rack and turned at 8 revolutions per hour at 37oC. After 24 hours the rings were checked for ciliary movement. Those showing poor or no ciliary activity were discarded.

5. Serology

Blood samples were collected from the brachial veins of 5 chickens from each infected group and from 2 chickens of the control group at 14, 21 and 28 days p.i. Serum antibody levels were measured using enzyme-linked immunosorbent assay (ELISA).

6-Enzyme linked immunosorbent assay (ELISA)

The indirect ELISA was used to measure anti IBV specific isotopic antibody responses in serum as described by Cook and Hugein (1986). An indirect ELISA was used to measure anti IBV specific isotypic antibody responses in serum and local secretion as described by Cook and Mockett (1986). ELISA plates were coated with optimally diluted purified IBV antigen in 50mM carbonate-bicarbonate buffer (PH 9.6) overnight at 40C. IBV coated wells were blocked with PBS contain 1% bovine serum albumin. Doubling dilutions of serum or secretion samples were prepared in PBS-contain 0.05% Tween/20) and added to the wells of virus-coated plate for one hour at 370C. After washing, virus specific chicken isotypic antibodies were detected employing optimally diluted isotype specific murine monoclonal antibodies against chicken IgM, IgG and IgA (kindly provided by Dr T.F. Division Animal Health Compton Laboratory, Newbury, Berks) for 1 hour at 370C. These were followed by application of goat antimouse IgG peroxidase conjugate (Sigma) for one hour at 37°C. Enzymic reaction was visualized by O phenylendiamine (OPD) as substrate (2.5 mM in 0.1 M carbonate phosphate buffer, PH 5.0 with 0.015% H2O2). After 10-15 minutes, the reaction was stopped by 50µl of 2.5 N sulphuric acid and plates were read at 490nm in a micro- ELISA reader (Dynatech). Between each individual incubation steps, the ELISA plates were washed each time six times with PBS-Tween20. Checkerboard titrations were performed to determine optimal dilutions of IBV antigen, monoclonal antibodies against chicken IgG, IgM and IgA and anti-mouse IgG peroxidase conjugate to be used in the test proper. A volume of 50µl /well of reagent was used throughout the test.

7. Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using viral RNA and a pair of oligonucleotides spaced 400 bases apart within a conserved region of the spike protein gene of infectious bronchitis virus. Dry tracheal swabs or swabs from chopped tissue collected at autopsy were pooled and processed by immersion in 400 µl of guanidine isothiocyanate (Solution D) and 2-mercaptoethanol to lyse the cells. The mixture was vortexed and left to stand at room temperature for 10 seconds. Next, 500 µl of a 10:3 mixture of phenol and chloroform (pH 7.5) was added to each tube, and the samples were vortexed vigorously for 10 seconds. The phases were then separated by centrifugation for 5 minutes, and the supernatant was transferred to a fresh tube containing 400 µl of 3M sodium acetate (1:10 volume) and 900 µl of cold absolute ethanol. The mixture was briefly vortexed, stored at –20°C overnight, and centrifuged for 5 minutes to pellet the precipitated RNA. The

supernatant was discarded, and the pellet was air-dried before being resuspended in 50 µl of sterile deionized water. One µl of this solution was used as the template for each PCR reaction.

8. T-cell suppression

T-cell suppression was induced by the application of CSA at a concentration of 100 mg/ml in maize oil (Neoral, Sandoz Ltd, Bordon, Hants, UK). The drug was injected intramuscularly into the thigh or breast muscle at a dosage of 100 mg/kg body weight every three days, as previously established by Dhinakar Raj and Jones (1996).

9. Virus isolation and titration

Aseptically collected tissues from euthanized birds were processed using the method described by Cook et al. (1991) for virus isolation and titration in tracheal organ cultures (TOC).

Results

1.Clinical signs

During the acute phase, clinical signs of IBV infection became evident within 72 hours post-infection (p.i.), persisting until day 10 p.i.. The observed symptoms were typical of IBV and aligned with previous reports. Following CSA treatment at 5 weeks p.i., a minority of birds showed very mild respiratory symptoms, including faint râles and clear nasal discharge, which was noticeable upon nasal squeezing.

2.Virus isolation and RT-PCR detection

Virus isolation results from TOC samples are summarized in Table 1. Each result represents a pool of five birds. Virus was successfully isolated from all sampled tissues for up to 10 days p.i., but not beyond day 14 p.i.. During the post-acute phase (days 21–35 p.i.), no virus was detected. However, following the initiation of CSA treatment at 34 days p.i., all tissue samples remained negative, except for kidney tissue at day 46 p.i., 12 days post the beginning of CSA treatment and after 3 passages.

RT-PCR was conducted on the re-isolated virus (M41). Agarose gel electrophoresis (Figure 1) revealed a single 400-base product, identical to that obtained from the M41 RNA, confirming the presence of IBV.

Table 1. Virus isolation in TOC from pooled tissues from chicks infected with IBV strain M41 when day-old and treated with CSA from 34 days of age.

Days post infection								
Tissue	3	7	10	14	21-35 [c]	46	49	52-55
Trachea	2 [a]	2	2	0 [b]	0	0	0	0–
Lung	2	2	2	0	0	0	0	0–
Kidney	3	2	2	0	0	3[d]	0	0–
B. of Fabricius	3	3	3	0	0	0	0	0–
Thymus	3	3	3	0	0	0	0	0–
Brain	3	**3**	3	0	0	0	0	0–
Rectum	2	2	2	0	0	0	0	0–

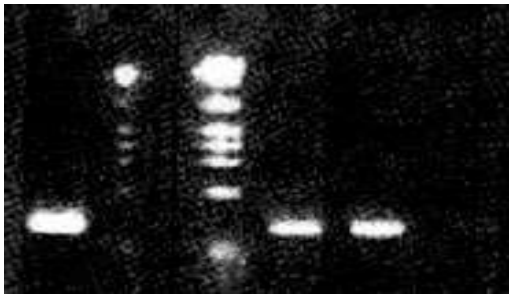
[a]: virus isolated from tissue pool; numbers indicate number of passages needed
[b]: no virus isolated
[c]: CSA injections were given from day 34-p.i., every 3 days for 15 days
[d] positive after 3 passages

RT-PCR. Results are shown in Table 2. In the acute phase, all tissues, which were positive by VI, were also positive by RT-PCR and both methods appeared to have similar sensitivity. No virus was detected between days 21-35. However, at day 46.p.i., i.e. 12 days after the start of CSA treatment, virus was detected in lung and kidney. All other subsequent samplings were negative.

Table 2. Virus detection by RT-PCR in the pooled tissues of chicks infected with IBV strain M41 when day-old and treated with CSA from 34 days of age.

	Days post infection							
Tissue	3	7	10	14	21-35[c]	46	49	52-55
Trachea	+[a]	+	+	–[b]	–	–	–	–
Lung	+	+	+	–	–	+	–	–
Kidney	+	+	+	–	–	+	–	–
B. of Fabricius	+	+	+	–	–	–	–	–
Thymus	+	+	+	–	–	–	–	–
Brain	+	+	+	–	–	–	–	–
Rectum	+	+	+	–	–	–	–	–

[a:] positive sample; b: negative
[c:] CSA injection were given from day 34-p.i., every 3 days for 15 days



A B C D

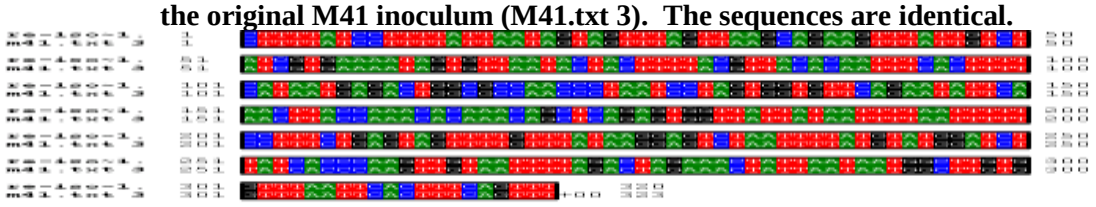
Figure.1. Agarose gel electrophoresis of the products of the RT-PCR.

Lane A: Positive IBV band from the kidney of a chick at 46 days of age. The chick was infected at day-old and treated with CSA from 34 days p.i.
Lane B: Molecular weight markers.
Lanes C and D: IBV M41 inoculum and a field strain of IBV, respectively.

Sequencing of the spike gene of the virus detected at 46 days

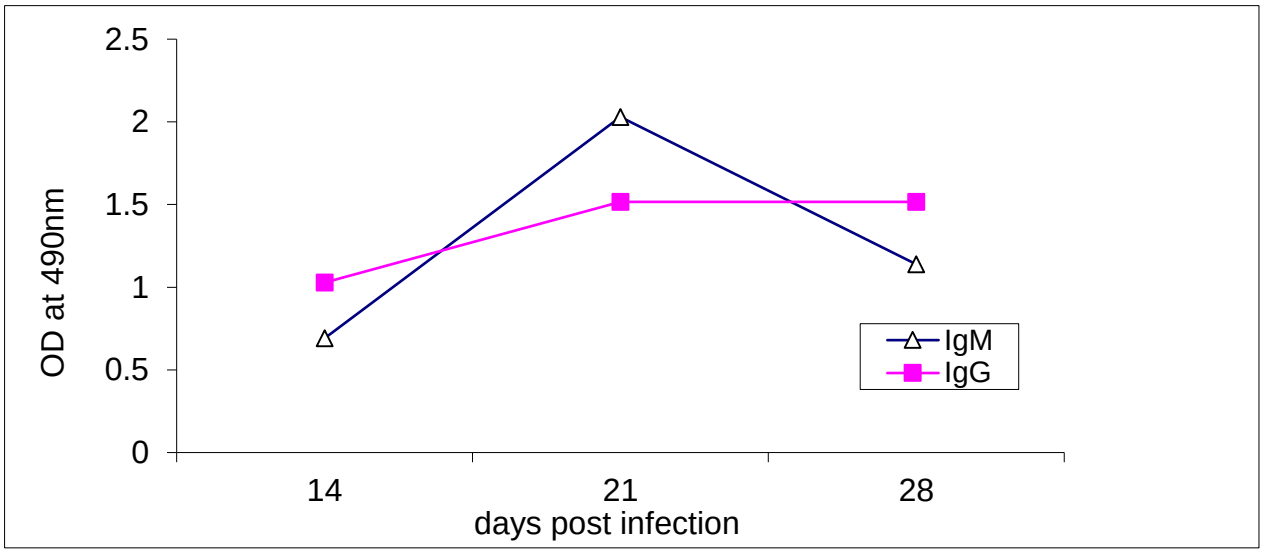
The PCR product from the kidney at day 46 was sent to Imperial College London for sequencing. The nucleotide sequences of the re-activated virus and the original M41 inoculum were found to be identical (Figure 2).

Figure.2. Nucleotide sequences of the S1 glygoprotein of the virus detected at 46 days pi (re-isol~1) and the original M41 inoculum (M41.txt 3). The sequences are identical.



Serum antibody responses:
Isotypic antibody responses against IB virus in chickens infected with IBV strain M41 are shown in Figure 3. Depicts the IBV-specific IgG and IgM profile in the serum of chickens infected at one day old with IBV- strain M41. IgG was present in significant amounts in the serum at day 21 p.i and dropped down by day 28p.i. IgM less significant compare to IgG on day 21 and this result was similar to a previous study (Dhinakar Raj and Jones 1997)

Figure 3. Virus-specific IgM and IgG in the pooled serum of chickens infected at one day old with M41, before treatment with CSA.



Discussion
Cyclosporin A (CSA) is an immunosuppressive agent that is widely used in transplantation. Animal studies indicate that CSA can affect the development of immunity so that autoreactive T lymphocytes are generated (Fukuzawa et. al., 1989).
In some animal viruses, reactivation is considered a sporadic event, occurring either spontaneously or due to external stimuli or stress factors that disrupt the equilibrium of an infected individual. These stimuli may be exogenous or endogenous, arising naturally (e.g., hormonal changes during reproduction) or induced artificially (e.g., corticosteroid treatment) (Elhafi, G. E *et al.,* 2024). Additionally, some animal viruses are known to undergo re-excretion episodes under specific stress conditions, such as re-housing (Gaskell and Povey, 1977; Vindevogel et al., 1985).
Re-excretion of IBV strain G has also been reported despite the presence of high titers of neutralizing antibodies (Ambali and Jones, 1990; Cook et al., 1968). Attempts to induce early sexual maturity and virus re-excretion by injection of estrogen and progesterone following early IBVG infection were unsuccessful (Ambali and Jones, 1991). However, CSA treatment administered at four weeks post-infection successfully induced virus re-excretion in most infected birds (Dhinakar Raj and Jones, 1997). CSA acts within hours of an activation stimulus. If administered within one hour of stimulation, it completely inhibits T-cell proliferation. However, if given more than six hours after activation, the effect on T-cell proliferation is minimal (Stuart *et al.,* 1992).
CSA primarily inhibits the production of interleukin-2 and other lymphokines, leading to a reduction in sensitized cytotoxic T-cell production and diminished antibody formation against T-cell-dependent antigens, without abrogation of humoral and reticuloendothelial immune responses (Elhafi, G. E *et al.,* 2024). In this study, birds received a CSA dosage of 50 mg/kg body weight every three days for five administrations (Dhinakar Raj and Jones, 1996). In contrast, other studies have administered CSA twice daily (Lillehoj, 1987; Corrier *et al*., 1991). It is possible that a higher dosing frequency could have resulted in greater viral excretion; however, intramuscular administration of CSA is likely painful for the birds, which may explain why this approach was not pursued.
The results confirm that infection at day-old leads to viral persistence, not only with enterotropic IBV strains (Bhattacharjee *et al*., 1995) but also with 'conventional' strains such as M41. Based on the findings of this experiment, it is reasonable to conclude that the kidney serves as the primary site of IBV persistence. The virus targets tubular epithelial cells within the kidneys, an immunologically privileged site that provides an optimal environment for viral persistence (Chong and Apostolov, 1982; Mimms, 1988).

These findings align with previous research by Dhinakar Raj and Jones (1997), despite differences in the IBV strain used. Sequencing analysis of the re-isolated virus confirmed its genetic similarity to the original strain, indicating that no mutations occurred during viral persistence in the kidney—at least within the examined genomic region.

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