The role of mucosal antibody in immunity to infectious laryngotracheitis virus in chickens

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The role of mucosal antibody in recovery from a primary infection and resistance to reinfection with infectious laryngotracheitis (ILT) herpesvirus was studied in bursectomized chickens, which were unable to synthesize specific antibodies. Viral antigen in the infected trachea was assessed by indirect immunofluorescence on tissue sections and by ELISA. The ability of bursectomized chickens to resolve primary infections as effectively as intact chickens and of vaccinated–bursectomized chickens to prevent the replication of challenge virus without the participation of mucosal antibody, is evidence for the importance of local cell-mediated rather than humoral immune mechanisms in the outcome of infection with ILT virus.

Introduction

Infectious laryngotracheitis (ILT) virus is an alpha-herpesvirus which causes a highly contagious respiratory disease in chickens (Jordan, 1981). The virus causes sporadic outbreaks of severe disease, presumably due to its ability to persist in a latent state in previously infected birds. Pathogenesis studies indicate that the virus causes a highly localized infection in the respiratory tract (Hanson, 1984). Viraemia and infection of visceral tissues occurs infrequently (Purcell & McFerran, 1969) and then usually in young chickens (Bagust et al., 1986).

Recent studies on the role of local immunity in the recovery of chickens from ILT found a correlation between the disappearance of viral antigen from the trachea and the appearance of mucosal antibody (York et al., 1989). This finding contrasted with previous studies which showed that bursectomized chickens, which were unable to synthesize specific antibodies, could be protected against clinical disease by vaccination (Robertson, 1977; Fahey et al., 1983). Although cellular immunity may prevent clinical ILT and mortalities in vaccinated chickens (Fahey et al., 1984) the question of whether mucosal antibody assists in the clearance of a primary infection from the trachea, or plays a role in preventing the establishment of a secondary infection remains unanswered.

This study records the rate of recovery from a primary infection with ILT virus in bursectomized chickens unable to synthesize mucosal antibody and also the ability of challenge virus to establish an infection in the tracheal epithelial cells of vaccinated chickens lacking mucosal antibody. The insights gained into the role of mucosal antibody in ILT are discussed.

Methods

Chickens. Specified pathogen-free (SPF) White Leghorn chickens of the CSIRO-W line produced by the CSIRO SPF Poultry Unit, Maribyrnong, Victoria were used throughout. The chickens were housed in flexible plastic isolators and fed fumigated feed and acidified water ad libitum.

Bursectomy. A combination of hormonal treatment in ovo and cyclophosphamide injections after hatching was used for bursectomy (Kowalski et al., 1978; Rose & Hesketh, 1979). Twelve-day-old embryonated eggs were injected via the allantoic sac with 3.5 mg of testosterone propionate (Sigma) in 0.1 ml sterile corn oil. Each chick was injected intraperitoneally with 2 mg cyclophosphamide (Sigma) at days 1 and 2 after hatching and with 3 mg on day 3 after hatching.

Viruses. SA-2, the vaccine strain of ILT virus used in Australia (Arthur Webster Pty Ltd, Castle Hill, Australia) and CSW-1, a virulent isolate of ILT virus, were propagated and assayed in primary chicken kidney (CK) cells as described previously (Fahey et al., 1983).

Detection of antibody by ELISA. The ELISAs for the detection of antibody were performed essentially as described by York et al. (1989) except that the antigen used was the SA-2 strain of ILT virus in tissue culture supernatant harvested 20 h after infection of CK cells. The medium was centrifuged at 1000 g for 10 min and the supernatant stored at −70 °C.

Polyvinyl chloride microtitre plates (Dynatech Laboratories) were coated overnight with rabbit IgG antibody to ILT virus. Antigen was added at a dilution of 1:5 followed by serum or tracheal washings at initial dilutions of 1:20 and 1:5 respectively. Affinity-purified goat anti-chicken IgG (heavy and light chains) conjugated to horseradish peroxidase (HRP; Kirkegaard and Perry Laboratories) was used at a dilution of 1:1000 to detect total antibody. Hybridoma culture supernatant containing monoclonal antibody to chicken IgA and
affinity-purified goat anti-mouse IgG conjugated to HRP (Tago) were used at dilutions of 1:100 and 1:1000 respectively to detect IgA antibody. The substrate was recrystallized 5-aminosalicylic acid (5-AS; Merck). The titre was taken as the highest dilution that gave an absorbance reading more than three standard deviations above the mean reading of samples obtained from uninfected chickens.

Detection of antigen by ELISA. A monoclonal antibody against ILT virus was used to detect viral antigen as described by York & Fahey (1988). Briefly, 150 µl volumes of undiluted tracheal washings were added in duplicate to polyvinyl chloride microtitre plates coated with rabbit antibody to ILT virus. Hybridoma culture supernatant containing monoclonal antibody against ILT virus (designated 131-24) was diluted 1:50 and added to the plates. The monoclonal antibody was followed by affinity-purified goat anti-mouse IgG conjugated to HRP (Tago) at a dilution of 1:100 and then 5-AS. An absorbance reading, corrected for background, of greater than 0.1 was considered positive.

Histology. Sections prepared from formalin-fixed tissues were stained with haematoxylin and eosin. Frozen sections were fixed in acetone for 5 min after cutting and stored at −70 °C until examined.

Immunofluorescence. IgA- and IgG-synthesizing cells present in frozen tracheal sections were quantified by incubating the sections with monoclonal antibodies against IgA (Southern Biotechnology Associates) and IgG (Australian Monoclonal Development) as described by York et al. (1989). Individual fluorescing cells in the tracheal epithelium and connective tissue of the whole section were counted; two sections from each chicken were counted.

Viral antigen in frozen tracheal sections was detected in a similar manner, using undiluted monoclonal antibody culture supernatant (131-24) followed by sheep anti-mouse IgG conjugated to fluorescein at a dilution of 1:25 (Silenus Laboratories). The level of replication in each section was quantified by giving a score from 0 to 10 to the proportion of the area of the section that contained fluorescing cells. The score was based on the area affected, not the intensity of fluorescence in any region of the section.

Statistical analysis. The ELISA absorbance readings for viral antigen were analysed using a one-way analysis of variance.

Experimental design. A total of 99 4-week-old bursectomized and intact chickens were vaccinated intratracheally with 1.5 × 10^3 p.f.u. of SA-2 ILT vaccine in a 100 µl volume. At days 0, 3, 5, 6, 7 and 8 post-infection (p.i.), groups of five chickens were killed by asphyxiation with CO₂. The thymuses and the bursal remnants in the bursectomized chickens were fixed in formalin. The thymuses were removed aseptically and the ends of the trachea were closed with surgical suture, which also secured a syringe and needle containing 1 ml sterile phosphate-buffered saline (PBS) into the bottom opening of the trachea. The trachea was washed by squirting the PBS in and out of the trachea several times and by manually massaging the trachea. The trachea was opened longitudinally and scraped with a scalpel. The tracheal scrapings were added to the washings which were then sonicated using an ultrasonic processor (Sonics and Materials). PMSF was added to a final concentration of 1 mM. The washings were centrifuged at 12000 r.p.m. for 2 min and stored at −70 °C until assayed.

At day 28 p.i. blood and tracheal washings were collected from three chickens from each of four groups: vaccinated-intact and vaccinated-bursectomized chickens and age-matched unvaccinated-intact and unvaccinated-bursectomized chickens. Both vaccinated and unvaccinated groups of chickens were challenged by intratracheal inoculation of 5 × 10^4 p.f.u. of CSW-1 in a 100 µl volume. Serum and tracheal washings were collected from eight to 10 chickens from each of the four groups at day 3 post-challenge, the time of maximum viral replication detectable by immunofluorescence (J. J. York, unpublished data) and the time after which viral antigen detectable by ELISA decreases (York et al., 1989). Bursal remnants collected from the bursectomized chickens at post mortem were fixed in formalin. Frozen tracheal sections were also prepared from the upper 0.5 cm of the trachea of three to five birds from each group at day 1 and day 3 post-challenge.

Results

The effect of bursectomy on a primary infection with ILT vaccine virus

Following vaccination with SA-2 ILT virus more than a third of the intact chickens showed signs of respiratory distress (gasping) on days 5 and 6 p.i., but none of the bursectomized chickens showed clinical signs. In total, six of the 44 (13-6%) intact chickens died following intratracheal vaccination, compared to two of the 70 (2-9%) bursectomized chickens.

The mean ELISA reading for viral antigen in the tracheal washings of the five bursectomized chickens examined post mortem at days 3, 5, 6 and 7 p.i. did not differ significantly from that detected in the five intact chickens at each of these time points (Fig. 1). In both groups the amount of viral antigen present in the tracheal mucosa declined from day 3 p.i. to reach undetectable levels by day 6 p.i. in the bursectomized chickens and by day 7 p.i. in the majority of intact chickens. Thus bursectomized chickens cleared the vaccine virus as effectively as the intact chickens.

When the tracheal washings from these chickens were assessed for the presence of total antiviral antibody by ELISA, none of the bursectomized chickens had detectable levels of humoral or mucosal antibody, even at day 8 p.i. (data not shown).

Histological examination of the thymuses and bursal remnants from each of the hormonally bursectomized chickens showed that the follicular structure of the bursae had totally collapsed, but the thymuses appeared unaffected. Over 50% of the treated chickens had no lymphocytes in the subepithelial regions. The remainder showed some repopulation, with from one to several small aggregations of lymphoid cells which showed no cortico-medullary differentiation.

The effect of bursectomy on the ability of vaccinated chickens to resist infection with virulent ILT virus

Immediately before challenge, both the vaccinated-bursectomized and vaccinated-intact chickens were negative for ILT viral antigen as measured by ELISA. At this time only the vaccinated-intact chickens had detectable levels of humoral and tracheal antibody to ILT virus (Table 1).

Three days after intratracheal challenge with virulent
ILT virus neither the vaccinated-bursectomized chickens nor the vaccinated-intact chickens had detectable levels of viral antigen in their tracheal washings, whereas unvaccinated-bursectomized and unvaccinated-intact chickens had readily detectable levels of viral antigen (Table 2). When frozen sections of the tracheas obtained from these chickens at 3 days post-challenge were examined by immunofluorescence, no cells infected with ILT virus were detected in the sections from either group of vaccinated chickens, both groups having a score of 0. Extensive viral replication was evident in the tracheal sections from both groups of unvaccinated chickens; the unvaccinated-bursectomized chickens had a mean score of 6-4, while the unvaccinated-intact chickens had a score of 6-7.

Only the vaccinated-intact chickens had detectable levels of humoral or mucosal anti-ILT virus antibody, a minor portion of which was IgA (Table 1). Frozen sections from the trachea of all four groups of chickens were also examined for the presence of IgA- and IgG-producing cells. Three days after intratracheal challenge with virulent ILT virus, immunoglobulin-synthesizing cells were detected only in the epithelial layer and lamina propria of the intact chickens (Table 3). In unvaccinated chickens IgA-positive cells predominated; in vaccinated chickens IgG-positive cells were most abundant.

To investigate the presence of a transient infection in the vaccinated-bursectomized and intact chickens, frozen tracheal sections were examined by immunofluorescence at 24 h after challenge with virulent ILT virus. No ILT viral antigen was detected in sections collected from a group of four vaccinated-bursectomized chickens, or three vaccinated-intact chickens, whereas four of five unvaccinated-bursectomized chickens showed low grade...
infections at that time, with less than 10% of the section containing infected cells.

Histological examination of the bursal remnants from hormonally bursectomized chickens showed that the subepithelial region of the majority of bursae contained lymphoid cells in small aggregations or follicles. These follicles showed no cortico-medullary differentiation.

**Discussion**

The role of cell-mediated immunity in recovery from herpesvirus infections and resistance to reinfection is well documented (Nash et al., 1985; Zarling, 1986), whereas the role of humoral or mucosal antibody is less clearly established (Kapoor et al., 1982b; Simmons & Nash, 1987). The concurrence of the disappearance of ILT virus from the trachea of infected chickens and the appearance of mucosal antibody (York et al., 1989) raised the possibility that mucosal antibody may play a role in recovery from infection. An extension of this finding would be that mucosal antibody plays a role in preventing reinfection of vaccinated chickens. The readily detectable levels of mucosal antibody found in the trachea following vaccination in both this and a previous study (York et al., 1989) provides support for such a hypothesis, particularly as virus-neutralizing activity has been detected in the tracheal washings of vaccinated chickens, albeit at low titre (Bagust et al., 1986; York et al., 1989).

Testosterone treatment *in ovo* and cyclophosphamide treatment post-hatching ablates the bursa of Fabricius and destroys the chicken's ability to synthesize specific antibody in response to antigenic challenge (Rose & Hesketh, 1979). In this study the hormonally bursectomized chickens failed to produce either humoral or mucosal antibodies to ILT virus following vaccination or challenge, even though there was some evidence of lymphoid repopulation of the treated bursae, particularly at 8 weeks of age. Similarly, bursectomized chickens had neither IgA- nor IgG-producing cells in the trachea after vaccination or challenge with ILT virus. Yet the bursectomized chickens recovered from a primary infection with ILT virus at a rate similar to that of the intact chickens. In fact there was an indication that antibody may contribute to the pathogenesis of disease since the bursectomized chickens appeared to have a less severe clinical reaction to the vaccine virus. The appearance of antibody in the trachea may affect the viscosity of the exudate which accompanies viral infection, thereby contributing to the onset of gasping and mortality due to asphyxiation.

More interesting, however, was the finding that the absence of mucosal antibody did not impair the ability of vaccinated-bursectomized chickens to resist totally a challenge infection with virulent ILT virus. As measured by immunofluorescence there was no evidence of a transient infection in these chickens at 24 h post-challenge. Furthermore there was no ILT viral antigen present at 3 days post-challenge, the time of optimal antigen production in unvaccinated-intact chickens (York et al., 1989). It would appear that in the case of ILT virus cellular, rather than humoral, immune mechanisms are able to protect the tracheal epithelial cells of vaccinated chickens from reinfection.

Evidence to date suggests that secretory IgA, and presumably other mucosal antibodies, protect the host by reacting with bacteria or viruses and preventing them attaching to or colonizing mucosal surfaces as was initially proposed by Freter (1969). In the absence of mucosal antibody therefore, pathogens may be able to establish infections before other specific and nonspecific protective mechanisms are invoked to limit the disease. In this study the failure to detect any replication of the challenge virus in vaccinated-bursectomized chickens does not support a role for mucosal antibody in vaccinal immunity to ILT virus.

It is not possible to conclude from the literature whether mucosal antibody plays a role in immunity to other herpesviruses, although Martin & Wardley (1987) reported that there was no correlation between mucosal antibody levels and protection of pigs against Aujeszky's disease. In humans there is no correlation between the presence of maternal antibodies to herpes simplex virus (HSV) and neonatal infection with HSV (Whitley et al., 1980). Similarly it has been reported that maternal IgG antibody does not confer resistance to infection with ILT virus (Cover et al., 1960; Jordan, 1981). However, immunization of female mice and oral feeding of newborn mice with hyperimmune human IgG antibodies has been shown to confer protection against an intraperitoneal infection with HSV (Hayashi et al., 1983). Experiments involving the passive transfer of monoclonal antibodies in mice also suggest that antibody may limit the spread of virus (Dix et al., 1981, Kapoor et al., 1982a; Simmons & Nash, 1985). Conversely, B cell-suppressed mice unable to make antibody cleared HSV as effectively as conventional mice (Kapoor et al., 1982b; Simmons & Nash, 1987).

Although much remains to be learnt of the immune mechanisms that resolve infections with ILT virus in chickens and those which enable vaccinated chickens to resist reinfection, it is apparent from this study that mucosal antibody does not have a central or critical role in recovery or vaccinal immunity. This may be explained by the intracellular nature of herpesvirus infections and the ability of the virus to spread from cell to cell, but our inability to detect even a transient low grade or focal
challenge infection in the tracheal epithelial cells of antibody-negative vaccinated chickens points to the efficacy of these antibody-independent mechanisms. Whether protection of the epithelium is mediated directly by cytotoxic lymphocytes, or by soluble factors (lymphokines) released by activated T cells, or both, remains to be answered.

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References


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