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Effect of in ovo bursectomy on the course of an infectious bronchitis virus infection in line C White Leghorn chickens

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Summary. White Leghorn line C chicks were surgically bursectomised (Bx) in ovo to eliminate antibody production. After inoculation with infectious bronchitis virus (IBV) at 14 days after hatching, Bx chicks experienced a more severe and longer lasting infection than intact chicks. The severity and duration of clinical infection in the Bx chicks resembled that previously observed in the highly susceptible line 15 I chicks, however no increase in mortality was observed, in contrast to the high levels of mortality recorded in IBV-inoculated line 15 I chicks. After secondary challenge the degree of damage to the ciliated epithelium of the trachea was greater in the Bx chicks than in the intact chicks.

The results indicate that, although antibodies play an important role in recovery from IBV infection, other immunological factor(s) may also be involved.

Introduction

During studies into genetic differences in the susceptibility of several lines of chicken to infection with infectious bronchitis virus (IBV) inbred lines of White Leghorn chickens were found to show considerable variation in mortality following intranasal inoculation with IBV alone [2]. In further work, a chicken line in which high mortality occurred (line 15 I) and one (line C) in which no mortality was recorded were selected and a number of different parameters of the disease, such as clinical signs, damage to tracheal epithelium and virus growth in tissues compared [15]. It was found that, whilst both lines were equally susceptible to infection initially, recovery was much more rapid in the line C chicks. Histological studies [14] showed that the type of damage which IBV infection caused to the mucociliary system of the respiratory tract was

similar in both lines but that the damage was more severe and longer lasting in line 15 I. This was confirmed by ultrastructural studies and by histochemical methods, which showed that IBV antigens could be detected in paraffin embedded tracheal sections of line 15 I chicks for longer than line C chicks. The finding that explants of tissues from the two lines were equally susceptible to in vitro inoculation with IBV led to the conclusion [15] that resistance to IBV infection was not due to lack of specific viral receptors, as is the case with murine hepatitis coronavirus [1], and it was suggested that immunological factors may be involved.

A number of approaches are currently being taken to investigate the importance of different aspects of the immune system on the rate at which chicks recover from IBV infection. One of these, the results of which are reported here, has been to surgically ablate the bursa of Fabricius in ovo and so deplete the chick of antibody producing lymphocytes before the peripheral lymphoid organs have been seeded. This technique has allowed us to compare the severity and duration of infection in line C chicks which were either able or unable to produce specific antibodies against IBV.

Materials and methods

Chickens

Chickens from two inbred White Leghorn lines, Reaseheath line C or East Lansing line 15 I [2], were used.

Embryonal bursectomy

Embryos were bursectomised on day 17 of incubation using a surgical technique [11]. In a preliminary experiment 4-week-old bursectomised (Bx) and intact chicks were injected with sheep red blood cells (SRBC) or *Brucella abortus* antigen. Samples of blood were obtained to determine the presence of B lymphocytes using an indirect immunofluorescent antibody (IFA) staining technique with a rabbit anti-bursacyte serum or rabbit anti-chicken immunoglobulin serum. Serum obtained after 7 days was used in micro-agglutination tests to detect the presence of antibodies to SRBC and *B. abortus*. There was complete agreement between the tests. Those chickens which had been completely bursectomised lacked circulating B-cells and were unable to develop an antibody response to either SRBC or *B. abortus*. Therefore in these experiments the efficacy of bursectomy was assessed by measuring circulating antibodies against IBV using an ELISA (see later). Those birds with evidence of circulating antibody were rejected from the experiments.

Experimental procedure

Groups of 14-day-old chicks which were intact or which had been bursectomised in ovo were inoculated intranasally (i.n.) with IBV using either the Massachusetts (M 41) strain [3] or a pool of virulent IBV strains all of the Massachusetts serotype (the IB Pool) [19] so that chicks received between $\log_{10}$ 4.6 and 5.7 median ciliostatic doses (CD_{50}) of virus in 0.1 ml. Following IBV inoculation intact and Bx chicks were housed in separate wire-floored pens in the same room in controlled environment accommodation. Uninoculated intact and Bx chicks acted as controls and were housed separately.

Chicks were examined regularly for clinical signs of infection. At intervals after inoculation four chicks from each group were bled from the wing vein and killed by intravenous

injection of sodium barbitone. Trachea, lungs, spleen and kidneys were removed aseptically and virus recovery attempted. Each organ was weighed, homogenised with sterile sand and a 10% w/v suspension prepared in Eagle's MEM containing 200 U/ml of penicillin and 200 µg/ml streptomycin. After standing for 60 min at 4 °C, suspensions were centrifuged for 20 min at 2000 **g** and serial 10-fold dilutions prepared in Eagle's MEM. These were titrated in tracheal organ cultures (OC) as described previously [3], end points being calculated by the method of Reed and Muench [17]. In addition, tracheal explants prepared from the same chicks were observed for ciliary activity as described previously [15].

Serology

Sera were examined for IBV-specific IgG antibodies using an ELISA [13]. In addition, sera from Bx chicks were examined for total immunoglobulins specific to IBV. In this ELISA, sera diluted 1:20 were added to IBV-coated plates. A rabbit anti-chicken total Ig conjugate (Nordic) was then added (1:400) followed by a goat anti-rabbit conjugate (ICN Immuno Biologicals) labelled with alkaline phosphatase (1:1000). The substrate and reading was as described previously [13].

Secondary challenge

In one experiment, groups of intact and Bx chicks which had been inoculated i.n. with the IB Pool at 14 days of age, were rechallenged 34 days later, together with previously uninoculated intact and Bx chicks, again with the IB Pool ($\log_{10}$ 4.6 CD_{50}/bird). Four and 7 days later, half the chicks in each group were killed, their tracheas carefully removed and explants examined for ciliary activity as decribed above.

Results

Mortality

Mortality was recorded following i.n. inoculation of intact and Bx lines 15 I and C chicks with the IB Pool. Although numbers in each group were small, bursectomy did not appear to increase the level of mortality observed in line

Table 1. Clinical signs of respiratory infection in bursectomised or intact line C chicks following inoculation with IBV

Days after inoculation	Clinical signs following inoculation with			
	M 41		IB Pool	
	Bx	intact	Bx	intact
2	++	++	++	−
5	++	++	+	+
9	+	+	+	±
12	−	−	+	−
16	−	−	±	−
20	−	−	−	−

Bx Bursectomised
++, +, ± Severity of respiratory signs
− No respiratory signs

15I chicks, which was 5/13 (38%) in the intact group and 4/7 (57%) in the Bx group. Little or no mortality was observed in line C chicks whether or not they had been bursectomised (0/39 in the intact group and 2/26 in the Bx group).

Clinical signs

Line C chicks were examined regularly for signs of respiratory infection following inoculation with M 41 or the IB Pool. The results summarised in Table 1 show that following inoculation with the IB Pool clinical signs were more severe and longer lasting in the Bx chicks than in the intact chicks. Respiratory signs were less severe following inoculation with M 41 and were similar in both groups.

Post mortem findings

The presence of nasal and tracheal exudate in the chicks killed at intervals after inoculation was also recorded. The results summarized in Table 2 showed that exudate persisted for longer in the Bx than the intact chicks inoculated with either M 41 or the IB Pool.

IB virus recovery from tissues

The results of attempts to recover virus from organs at intervals after IBV inoculation are summarized in Fig. 1 which shows the median value for the 3 or 4 observations at each time. In the case of M 41, there was a clear cut difference between the Bx and intact chicks in the duration of virus recovery from the trachea and lungs. With spleen and kidney differences were evident

Table 2. Nasal and tracheal exudate following inoculation of intact and bursectomised line C chicks with IBV

Days after inoculation	Exudate following inoculation with			
	M 41		IB Pool	
	Bx	intact	Bx	intact
2	4/4	4/4	2/3[a]	1/3
5	4/4	4/4	2/3	3/3
9	1/3	2/4	4/4	4/4
12	0/3	1/4	3/4	2/4
14	4/4	1/4	NE	NE
16	1/3	0/4	2/4	0/4
20	1/4	0/4	3/4	0/4
23	0/4	0/4	2/3	0/4
28	1/3	0/4	1/3	0/4

[a] Number with exudate/number killed
NE Not examined

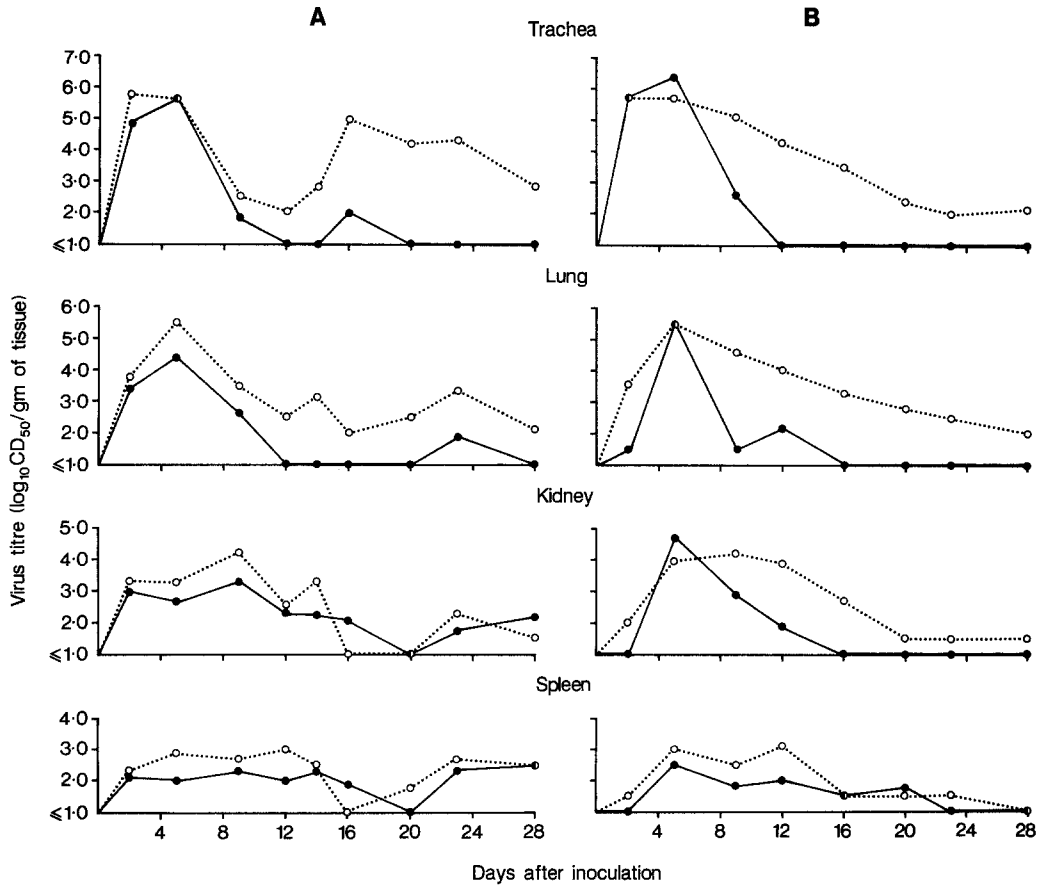

Fig. 1. Recovery of IBV from tissues of intact (● – ●) and bursectomised (○···○) chicks at intervals after inoculation with the M 41 strain (**A**) or the IB Pool (**B**). Median values are shown for the 3 or 4 chicks examined at each time

initially, but these were less marked. In the case of the IB Pool, overall more virus was recovered and for longer from each tissue of the Bx than the intact chicks. In a third experiment (inoculated with M 41) virus recovery from the Bx and intact groups was similar and amounts of virus recovered were appreciably lower than in either of the other two experiments (data not shown).

Ciliostasis test

The results of examining tracheal explants prepared from intact and Bx chicks at intervals after inoculation with each IBV strain are shown in Table 3. Initially, following inoculation with either strain, similar levels of ciliostasis were observed in both Bx and intact chicks but the tracheas of the intact chicks recovered much more quickly than those of the Bx chicks.

Serology

All chicks which were to be killed during these experiments were bled and their sera examined for IBV antibodies by ELISA. Sera from the Bx chicks were

Table 3. Ciliary activity in tracheal rings prepared from intact and bursectomised line C chicks killed at intervals after inoculation with IBV

Days after inoculation	Activity score in chicks inoculated with			
	M 41		IB Pool	
	Bx	intact	Bx	intact
2	4.0	4.0	3.9[a]	2.4
5	4.0	4.0	4.0	4.0
9	3.7	3.5	3.9	3.6
12	2.0	1.6	3.7	1.2
14	2.8	1.5	NE	NE
16	2.5	0.8	3.6	0.1
20	3.4	0.7	2.5	0
23	2.4	0	1.5	0
28	1.9	0.3	0.6	0

[a] Mean score for the 10 explants from the tracheas of the 3 or 4 chicks examined at each time

NE Not examined

Table 4. Ciliary activity in tracheal explants prepared from intact and Bx line C chicks challenged with the IB Pool

Days after challenge	Mean activity score per explant			
	2nd IBV inoc.		1st IBV inoc.	
	Bx	intact	Bx	intact
4	2.1[a]	0.3	4.0	4.0
7	1.2	0.1	4.0	4.0

[a] Mean score for 10 explants from the tracheas of 3 chicks examined at each time

examined at a 1:20 dilution using a total Ig conjugate and any chick whose serum was found to show a positive reaction at this dilution was eliminated from the experiment. This was a total of 4 and 7 chicks in the experiments challenged with M 41 and 2 in the experiment challenged with the IB Pool. Sera from the intact chicks were assayed to determine titres of IBV-specific IgG antibodies. IgG was first detected 9 days after inoculation and peak titres of approximately 1:900 were reached by 3 weeks.

Secondary challenge

Intact and Bx chicks which had been inoculated with the IB Pool 34 days earlier, together with previously uninoculated intact and Bx chicks were challenged i.n. with the IB Pool. Clinical signs of respiratory infection were observed in the

two groups inoculated for the first time but not in the chicks receiving the secondary challenge. Three chicks in each group were killed after 4 and 7 days and their tracheas examined for ciliary activity. The chicks inoculated with IBV for the first time showed mean ciliostasis scores of 4.0, the maximum score possible (Table 4), indicating that they were fully susceptible to IBV infection. The low scores in the intact, rechallenged group indicated that they were protected against rechallenge, whilst the Bx, rechallenged chicks were found to be only partially protected.

Discussion

Mature B lymphocytes begin to migrate from the bursa of Fabricius and seed the peripheral lymphoid organs from day 18 of embryonic development [5]. Hence complete removal of the bursa on day 17 should result in a chick which lacks both B lymphocytes and the ability to generate specific antibody responses [5, 18; present study]. Surgical bursectomy at 17 days incubation is an efficient and specific way of depleting the B-dependent immune system without impairing cell-mediated immune responses. Incompletely bursectomised chicks can readily be identified by the presence of circulating antibody and in these three experiments only 13 out of 111 (11%) of those chicks bursectomised in ovo were discounted on this criterion. However, it must be pointed out that those chicks sampled on days 2 and 5 did not have a sufficiently high titre of antibody to allow this criterion to be used and the completeness of bursectomy in this case was confirmed by post mortem examination.

In this study, two different IBV inocula were used: the pool of virulent strains, which had been used previously and shown to cause high levels of mortality in susceptible chicks, when inoculated alone [2] or together with pathogenic serotypes of *Escherichia coli* [4, 19], and the M 41 strain [14, 15]. The M 41 strain had been selected because it was considered undesirable to cause high levels of mortality in chicks to be used for virus assays – such assays on moribund chicks being likely to be inaccurate. For the same reason, it was initially decided to use the M 41 strain in the present studies. However, in two experiments with that strain rather different results were recorded. In one of them only mild clinical signs were observed in both the Bx and intact chicks and virus was recovered from tissues from both groups in very small amounts and for only a short time. A third experiment was therefore carried out, challenging with the IB Pool. A more severe respiratory infection was recorded and amounts of IBV recovered and the duration of recovery were similar to that found in one experiment where M 41 was used. The reason for this discrepancy is not immediately clear, however, previous findings (H. W. Smith et al., unpubl.) have indicated that the severity of infection which the M 41 strain of IBV causes in chickens inoculated with IBV alone or together with pathogenic strains of *E. coli* can be highly variable.

In one experiment in which M 41 was used and in the experiment with the IB Pool, bursectomy had the effect of increasing both the duration and the

severity of the clinical disease caused by IBV in the line C chicks and of increasing the time for which virus could be recovered from tissues, thus making the course of the infection more like that previously observed in the line 15 I chicks [15]. The Bx line C chicks did not behave completely like line 15 I chicks, however, because negligible mortality was recorded in them, even after inoculation with the pool of virulent IBV strains, which consistently causes high mortality (greater than 80%) in the line 15 I chicks [2]. The reason for this is not clear but suggests that the B-system is not the only arm of the immune system involved in the differing behaviour shown by line C and line 15 I chicks following IBV challenge.

The mechanisms involved in immunity to IBV infections are complex and are still not fully understood. The trachea is the primary target organ for IBV and there is considerable evidence to suggest that local immunity in the upper respiratory tract is an important first line of defence against IBV infections [7–9]. The role of the Harderian gland in such immunity has been established [6, 7].

Chickens develop a good humoral response to IBV challenge, measurable by a number of different assays including ELISA, haemagglutination inhibition and serum neutralisation [12], yet there is much evidence to suggest lack of correlation between titres of circulating antibody and resistance to infection [10, 16, 21]. Although numbers of chicks in the secondary challenge experiment were small, it was clear that the degree of damage to the ciliated epithelium of the trachea in Bx chicks, which had been unable to mount a humoral response to the primary IBV inoculation, was much more severe than that in the intact chicks. This suggests that antibody may have contributed to the protection against IBV challenge.

The role of cell mediated immunity (CMI) in protection against or recovery from IBV infections has received little attention. In view of the lack of correlation found between humoral antibody and protection, it was suggested that CMI might be important and CMI responses were demonstrated in IBV-infected chickens [20]. However, the significance of CMI in resistance to or recovery from IBV infections has yet to be resolved.

The studies reported here suggest that circulating antibody may play a role in the differing rates of recovery of lines C and 15 I chicks following IBV infection but indicate that some other factor or factors are also involved. The role of locally produced immunity in the upper respiratory tract is currently under investigation, as is the importance of CMI.

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