Cloacal Inoculation with the Connecticut Strain of Avian Infectious Bronchitis Virus: An Attempt to Produce Nephropathogenic Virus by *in vivo* Passage Using Cloacal Inoculation

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ABSTRACT. Avian infectious bronchitis virus (IBV) strain Connecticut A-5968 isolated from respiratory tissue of chickens in USA in the 1960s, is considered as representative of respiratory disease causing IBV strains. Specific pathogen free chicks inoculated with the strain via the cloaca replicated the virus more rapidly in their kidneys than did chicks inoculated with the same virus intratracheally. Virus passaged thirteen-times via the cloaca caused stronger nephrotropism and nephropathogenicity than the parent virus. It is suggested that cloacal inoculation of IBV provides new and interesting information concerning the mechanisms of nephropathogenicity of IBV which has became increasingly important worldwide during the last ten years. — KEY WORDS: avian infectious bronchitis virus, cloacal inoculation, IBV, nephropathogenicity, nephrotropisum.

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Avian infectious bronchitis virus (IBV) has complex pathogenicity. Some IBV strains have virulence for the kidneys or oviduct in addition to the respiratory tract, and this pathogenicity have been considered to depend strongly on virus strain [6]. Jones [4] reported, however, that chickens inoculated with a non-nephropathogenic strain of IBV via some artificial routes showed nephrosis. He succeeded in producing nephrosis, by both intravenous and intra-oviduct inoculation of 30-week-old chickens, with the Massachusetts-41 strain of IBV which had been considered not to cause nephrosis. Nevertheless, natural infection via the intravenous or oviduct routes is unlikely to occur in the field. The necessary conditions for a strain of IBV to have a nephropathogenicity have not been sufficiently analysed and remain obscure.

It is well known that most field strains of IBV loose their virulence for chickens following repeated passage *in vitro* or *in ovo* [6]. Although IBV strains have several target tissues such as respiratory, reproduction and digestive ones, variation of organ tropism of the viruses has been poorly characterised. Otsuki *et al.* [10] attempted to change tissue tropism of IBV. They succeeded in removing tropism for the kidney from a nephropathogenic strain of IBV by 10 *in vivo* passages in respiratory tissue using tracheal inoculation, but failed to induce nephrotropism in a non-nephropathogenic IBV by passage in kidneys using intravenous inoculation.

A field strain of IBV HS-91, isolated from the kidneys of a chicken which died of nephrosis, produced more severe kidney lesions in a shorter period following cloacal inoculation and more virus were recovered from kidneys in comparison to those inoculated via the trachea [13]. Thus it can be considered that cloacal inoculation is an effective route to reproduce nephrosis/nephritis with IBVs that have strong nephrotropism. However, cloacal inoculation of non-nephropathogenic strain of IBV has not yet been investigated. In this study, IBV strain Connecticut A-5968 (A-5968) was used to determine whether or not it could infect chickens and produce kidney lesions when inoculated via the cloaca, since this IBV strain has been considered as a typical respiratory disease causing one [14]. IBV strain A-5968 was further investigated to try to change its organ tropism, that is, from respirotropism to nephrotropism by several cloacal passages.

MATERIALS AND METHODS

Virus: IBV strain A-5968 was used in this investigation [8]. This virus was supplied by the National Institute of Animal Health, Paultry Disease Laboratory , Gifu, Japan and was passaged ten times in specific pathogen free (SPF) embryonated hen's eggs in this laboratory.

Tracheal ring organ cultures: Tracheal rings of SPF chick embryos were produced by the method described by Cook *et al.* [1] and used for virus titration and recovery.

Experimental infections with strain A-5968 via the cloaca or trachea of SPF chicks

Experiment 1: Experiment 1 (Expt 1) was basically performed as described previously [13]. Sixty 4-day-old chicks were divided into two groups, groups C and R and each group contained 30 chicks. The chicks in group C were inoculated (0.1 m*l*) with IBV strain A-5968 $10^{5.8}$ median ciliostatic doses (CD_{50})/0.1 m*l* via the cloaca and group R were inoculated with the same volume of virus via

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the trachea. Another 30 chicks formed the negative control group and were inoculated with phosphate buffered saline via the cloaca. All chicks were deprived of food for more than 24 hr prior to inoculation. Each group of chicks was placed in a brooder housed in separate but similar rooms. Every day for eight days postinoculation (PI) three chicks from each group were examined for clinical signs, killed and necropsied. Respiratory tissues, including trachea and lungs, caecal tonsils with rectum (lower parts of intestine) and kidneys of each chick were then collected aseptically. These samples were stored at −35°C until examined for virus recovery.

Experiment 2: Six 4-week-old SPF chicks were divided into groups C and R and inoculated (0.2 m*l*) with IBV strain A-5968 ($10^{6.5}$ CD_{50}/0.1 m*l*) in the same manner as in Expt 1. The chicks were examined for clinical signs, bled and the serum separated at two and four weeks PI. Prior to assay for serum neutralising (SN) antibody, the sera were stored at −35°C.

Experiment 3: This experiment was designed to investigate the correlation between virus titre of inoculum and that recovered from kidneys in groups C and R. One hundred and thirty-two 4-day-old SPF chicks were divided into groups C and R, each of 66 chicks. IBV strain A-5968 ($10^{6.5}$ CD_{50}/0.1 m*l*) was diluted serially in 10 fold dilutions from 10^{-2} to 10^{-7} in PBS and 11 chicks of each group were inoculated with 0.1 m*l* of each virus dilution via the cloaca or trachea respectively. The inoculated chicks were housed strictly in separate cages, observed for clinical signs until 4 days PI, then killed and necropsied. Kidneys of the chicks were then collected aseptically and stored at −35°C until used.

Virus recovery: Virus recovery was attempted from the tissues by the method described by Cook *et al.* [1] and Nakamura *et al.* [7].

SN test: The SN test was performed by the method described by Cook *et al.* [2].

An attempt to produce nephrotropic or nephropathogenic IBV from Connecticut A-5968 strain of IBV

In vivo *passage of IBV in kidneys of chicks by cloacal inoculation*: Inocula were prepared from kidneys of chicks inoculated with IBV via the cloaca. The inoculum for the first passage was obtained from the kidneys of one chick which died in Expt 1, group C, showing gross kidney lesions. The kidneys were homogenised and 10% (w/v) suspended in PBS containing 1,000 IU/m*l* of penicillin and 1,000 μg/m*l* of streptomycin. After centrifugation for 5 min at 170 g, the supernatant was inoculated (0.1 m*l*/bird) into the cloaca of 20 4-day-old SPF chicks. The chicks were killed and examined postmortem at five days PI. The numbers of chicks showing kidney lesions were recorded. The kidneys showing some abnormalities were removed from three chicks and stored at −35°C until the next inoculation. The samples were then homogenised and inoculated into the cloaca of 20 4-day-old SPF chicks in the same manner as described above. The virus titre in each inoculum was determined. The virus was passaged a total of 15 times. The virus which had received 13 passages was found to induce the highest incidence of kidney lesion and mortality and was therefore selected to study tissue tropism and serological properties. It was passaged once in embryonated SPF eggs.

Tissue tropism of parent and passaged viruses: The titre of both the parent and passaged virus of strain A-5968 were adjusted to approximately $10^{6.5}$ CD_{50}/0.1 m*l* in PBS. Twelve 4-day-old SPF chicks were divided into groups I and II. Group I chicks were inoculated with the parent virus via the cloaca (group I_C) or via the trachea (group I_R). Group II chicks were inoculated with the passaged virus via the cloaca (group II_C) or via the trachea (group II_R). All chicks were killed and examined postmortem at 5 days PI. Respiratory organs and kidneys were collected aseptically and examined for virus recovery.

Serological comparison between parent and passaged virus: Both the parent and passaged viruses were inoculated into two groups of five 4-week-old SPF chicks via the cloaca. The chicks were reinoculated with the same viruses via the cloaca at 2 weeks PI, then bled at 4 weeks after the first inoculation and the sera separated. The sera of each group of five chicks were pooled. Pooled sera were tested for neutralising antibody titre against the parent and passaged virus.

Histopathological comparison of kidneys of chicks inoculated with parent and passaged viruses: Forty 4-day-old SPF chicks were divided into four groups and inoculated with the parent or passaged viruses via the cloaca or trachea. All chicks were killed at 5 days PI. Respiratory tissues and kidneys were dissected out and fixed in buffered formalin. Sections were cut in paraffin at 4 μm and stained with haematoxylin and eosin. For the objective evaluation of the severity of histopathological changes, the extent of tracheitis, degeneration of urinary tubules and inflammatory reaction in the interstitium of the kidney was expressed by pathological scores, 1 (mild), 2 (moderate) and 3 (severe), respectively.

Statistical analysis: The data were statistically analysed by Student's or Welch's *t*-test; $p<0.05$ was considered as statistically significant.

RESULTES

Experimental infection of SPF chicks with strain A-5968 via the cloaca or trachea (Expt 1)

(1) Clinical findings

Chicks inoculated with IBV A-5968 via the cloaca (group C) showed mild respiratory signs from 4 days PI, whilst the intratracheally inoculated chicks (group R) showed mild respiratory signs from 1 day PI and severe ones from day 2 (data not shown). In group C, two chicks died at 3 and 4 days PI, these showed airsacculitis and pale and swollen kidneys (Fig. 1A). In group R, three chicks which died 1, 2 and 5 days PI were affected by severe respiratory disease, but did not show any kidney lesions. The postmortem findings obtained from individual chicks which did not die

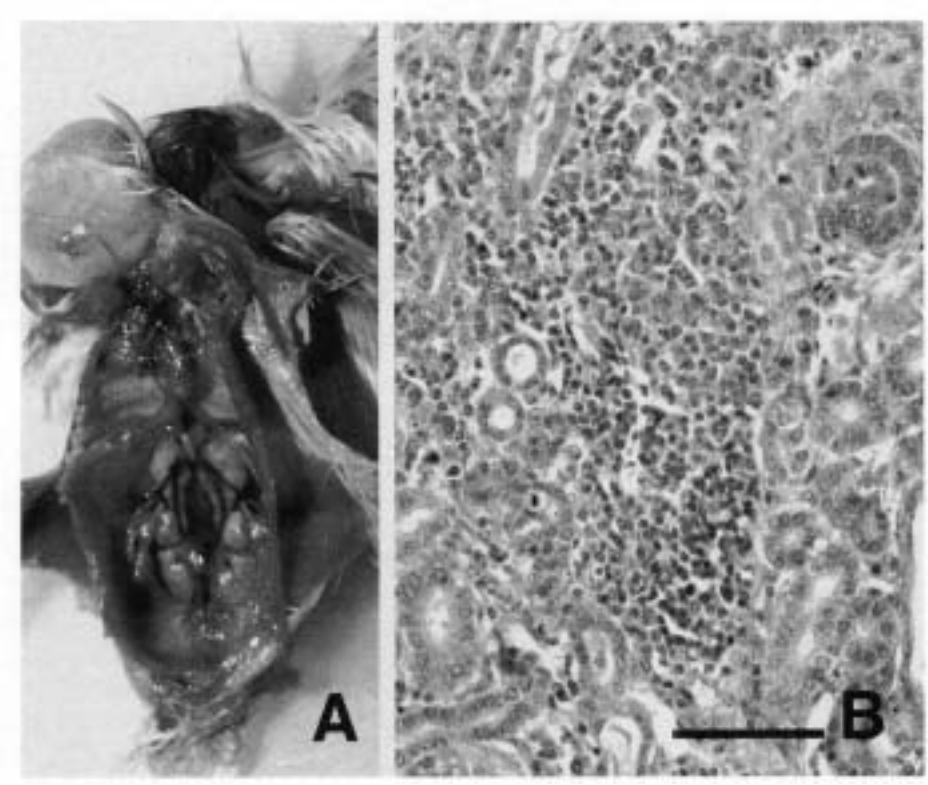

Fig. 1. A chick inoculated with IBV strain A-5968 via cloaca, showing pale and swollen kidneys (A). A pathological feature in the kidney of the cloacal inoculated chick showing a marked infiltration of lymphocytes into the interstitium (B). (HE, bar = 50 μm).

are summarised in Table 1. The five chicks that died during the experimental period are excluded from this Table. In group C, airsac lesions, including opacification of the air sacs and secretion of icteric like sputum in the air sac, were observed sporadically from 3 days PI, whilst in group R, these lesions were seen constantly from 5 days PI. Negative controls showed neither clinical nor postmortem findings. Remaining seven chicks were not examined in this investigation.

(2) Virus recovery

Virus was successfully recovered from the respiratory organs, kidneys and lower parts of the intestine of chicks in both groups. Geometric mean titres (GMTs) calculated for each group of three chicks are shown in Fig. 2. In the primary target organs (respiratory tracts in group R and lower parts of the intestine in group C), GMTs were high on the day following inoculation and almost unchanged throughout the experimental period. The virus titres in the target organs other than the primary target tissue were markedly different among chicks at each time, regardless of the type of inoculation. In group C, the virus titre in the respiratory organs increased after infection, reaching a maximum at 4 days PI and thereafter gradually decreased to the level at 1 day PI. GMTs in the intestine were always below the group R values during the postinoculation days, except at 8 days. In the kidneys, the viral titres increased after the cloacal inoculation and were maintained at high levels until 8 days, then dropped rapidly, whilst the titres following the tracheal inoculation reached a peak at 5 days, but the values varied throughout the postinoculation period.

Experiment 2

(1) Clinical findings

In group C chicks, no respiratory signs were evident during the experimental period, whilst in group R, respiratory signs appeared 1 day PI and lasted for a week.

(2) Production of SN antibody

SN antibody productions in groups C and R chicks are shown in Fig. 3. At 2 weeks PI, the antibody titre was significantly higher in group C chicks (1:125) than in group R (1:20) ($p<0.01$).

Experiment 3

(1) Clinical findings

Clinical signs and postmortem findings of groups C and R chicks inoculated with serial 10 fold dilution of IBV strain A-5968 are summarised in Table 2. In group C, kidney lesions were observed in one chick inoculated with a dose of $10^{2.5}$ CD_{50} and in three chicks inoculated with both $10^{3.5}$ and $10^{4.5}$ CD_{50} of virus (one chick in each group died with kidney lesions). Respiratory signs were only observed in group C chicks inoculated with $10^{4.5}$ CD_{50}. In group R chicks, respiratory signs were observed by 3 days PI when a dose of at least $10^{1.5}$ CD_{50} was inoculated. No kidney lesions was observed in any group R chicks. Two group R chicks inoculated with $10^{0.5}$ or $10^{4.5}$ CD_{50} died with no macroscopic

Table 1. Postmortem findings in chicks infected with IBV Connecticut A-5968 in Experiment 1

PI (days)	chick No.	Group C airsacculitis[a)]	Group C kidney lesions[b)]	chick No.	Group R airsacculitis	Group R kidney lesions
1	1	–	–	1	–	–
	2	–	–	2	–	–
	3	–	–	3	–	–
2	4	–	–	4	–	–
	5	–	–	5	–	–
	6	–	–	6	–	–
3	7	–	+	7	–	–
	8	+	–	8	–	–
	9	–	–	9	–	–
4	10	–	–	10	–	–
	11	+	–	11	–	–
	12	–	–	12	–	–
5	13	–	+	13	++	–
	14	–	–	14	+	+
	15	++	+	15	++	–
6	16	–	–	16	++	–
	17	++	–	17	–	–
	18	–	–	18	++	–
7	19	–	–	19	++	–
	20	++	–	20	++	–
	21	–	–	21	–	–
8	22	+	–	22	++	–
	23	–	–	23	++	–
	24	–	–	24	+	–

a) – No lesions; + Opacification of air sac; ++ Severe opacification of the air sac and secretion of icteric-like sputum in the air sac.
b) – No lesions; + Pale and swollen.

lesions.

(2) Correlation between viral inoculum size and amount of the virus recovered from the kidneys of groups C and R chicks

GMTs were examined in each group of 10 or 11 chicks inoculated with the serial 10 fold dilutions of IBV. The virus recovery in the kidneys increased in proportion to the inoculum size. In the chicks inoculated with $10^{4.5}$ CD_{50}, titres of recovered virus were significantly higher in group C than in group R ($p<0.05$) (data not shown).

There was correlation between viral inoculum size (CD_{50}/0.1 m*l*) and amount of virus recovery in 0.1 g of the kidneys (GMT) of each group as analysed separately, in groups C and R (Fig. 4). There was a significant correlation between the inoculum size and virus recovery (group C, r=0.9; group R, r=1.0).

An attempt to produce nephrotropic or nephropathogenic IBV from respirotropic Connecticut A-5968 strain of IBV

(1) Clinical findings, incidence of kidney lesions, mortality and virus recovery during passage

Figure 5 shows the incidence of kidney lesions, mortality and virus recovery from the kidneys of the chicks inoculated with each level of cloacally passaged virus. At each passage, the inoculated chicks were examined at 5 days PI. Kidney lesions were first seen in chicks inoculated with virus passaged twice via the cloaca in this attempt. Eighty percent of the chicks inoculated with 5th and 7th times passaged virus produced kidney lesions, whilst only 20% of the chicks inoculated with passage 9 virus did so. Thereafter, incidence of kidney lesions gradually increased with progressive passage. The amount of virus recovered was stable throughout this serial passage. A few chicks inoculated with the IBV passaged via the cloaca more than twice showed inappetence, listlessness, ruffled feathers and thirst, and died between 3 and 5 days PI. They had pale and swollen kidneys with distended renal tubules. Ten to 30% of the chicks inoculated with either parent virus A-5968 or the virus passaged 15 times via the cloaca showed respiratory signs from 4 days PI.

(2) Tissue tropism of parent and passaged viruses

Replication of parent and 13-time passaged viruses in both respiratory tissues and kidneys of SPF chicks were compared when these viruses were inoculated intratracheally or intracloacally. The amount of passaged virus recovered from the kidneys was approximately the same as that of parent virus when these viruses were inoculated into the trachea, whilst the passaged virus was recovered less well than the parent virus from the respiratory tract following the intracloacal inoculation (Fig. 6). When these viruses were inoculated intratracheally, there was no significant

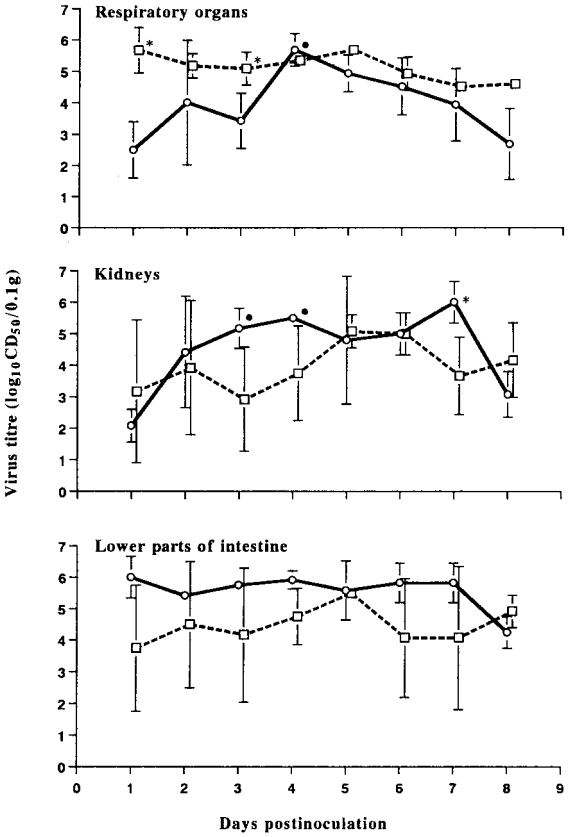

Fig. 2. Profile of titres of virus recovered from respiratory organs, kidneys and lower parts of the intestine in Group C (—○—) and Group R (---□---). * The value is significantly different from the other group. ($p<0.05$). ● The value significantly increased from the day 1 value for the group ($p<0.01$).

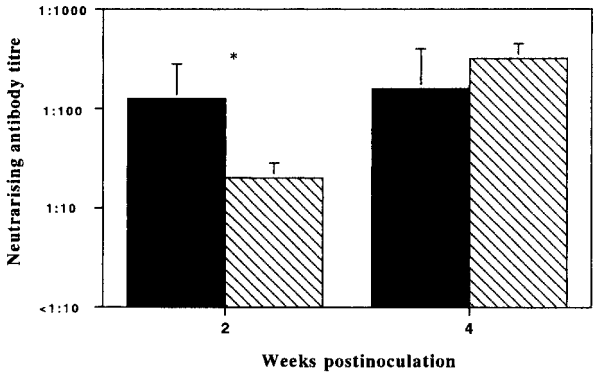

Fig. 3. Serum neutralising antibody titres in Group C (■) and Group R (▨). Vertical lines indicate standard deviations. * The values of week 2 are significantly different from each other ($p<0.01$).

difference in viral replication between respiratory tissues and kidneys.

(3) Serological comparison between parent and passaged virus

The antigenicity of the parent and 13-time-passed viruses were compared (Table 3). Marked antigenic variation did not occur during the passaging.

(4) Histopathological comparison between lesions caused by parent and passaged viruses

Histological changes caused by the IBV infection in the kidneys were swelling, vacuolar degeneration and necrosis in epithelial cells of renal tubules, especially in convoluted tubules (with or without infiltration of heterophils), and formation of spherical, eosinophilic crystals in the tubular lumen. Some of the collecting tubules were slightly dilated and contained debris of epithelial cells and leucocytes. Lymphocytes and plasma cells infiltrated into the interstitium of the affected kidney (Fig. 1B). In the lungs and trachea of the IBV-inoculated chicks, major pathological findings were catarrhal broncho-tracheitis characterized by loss of cilia, swelling to necrosis of the mucosal epithelial cells and infiltration of heterophils, macrophages and lymphocytes into the mucosa of the trachea and bronchi. None of the control chicks showed these lesions.

The severity of the histological lesion scores for each group is shown in Fig. 7. The renal lesions were more severe in cloacal inoculated chicks than in tracheal inoculated ones. When the virus was inoculated in the cloaca, the passaged strain was more pathogenic for the kidneys in comparison to the parent virus.

Table 2. Clinical signs and postmortem findings in Experiment 3

Size of inoculum (log_{10} CD_{50})	Group C			Group R		
	mortality[a]	respiratory signs[b]	kidney lesions[c]	mortality	respiratory signs	kidney lesions
−0.5	1	—	0	0	—	0
0.5	0	—	0	1	—	0
1.5	0	—	0	1	3	0
2.5	0	—	1/11	0	2	0
3.5	1	—	3/11	1	3	0
4.5	1	3	3/11	1	2	0

a) Number dead/11 in group.
b) — No respiratory sign detected in the chicks. Numbers (2 or 3) indicate the days PI when the signs were first detected in.
c) Number with kidney lesions of 11 in group.

DISCUSSION

It is known that field strains of IBV are divided into roughly two types, respiratory disease- and nephrosis-causing ones and it has been suggested that the nephrosis-causing IBV arose from that causing respiratory disease [10]. Jones [4] reported that chicks inoculated with the Massachusett-41 strain of IBV, which had been considered not to be associated with nephrosis, caused the kidney disease when it was inoculated intravenously or via the oviduct. A non-nephrosis-causing virus, A-5968, was demonstrated in the kidneys of chicks subjected to aerosol inoculation [3]. On the other hand, the chicks inoculated with a nephrosis-causing IBV, strain HS-91, via the cloaca showed more severe clinical signs, grosser kidney lesions and higher mortality than those inoculated via the trachea [13]. However, investigations of cloacal inoculation with non-nephrosis-associated IBV strains have not so far been performed. The present study revealed that strain A-5968 caused kidney abnormality, in addition to respiratory signs, only in chicks inoculated via the cloaca. Virus titre recovered from the kidneys reached high levels earlier after infection in chicks inoculated via the cloaca than in those inoculated via the trachea. These results indicate that the strain A-5968, as well as the nephrosis-causing strain HS-91, is capable of inducing kidney lesions, only when the virus is inoculated via the cloaca. This conclusion is in line with a hypothesis put forward by Uenaka *et al.* [13] that the cloaca is one of the most efficient natural inoculation routes to spread IBV to the kidneys. The cloacal inoculation of IBV has the ability to provide a larger amount of virus to the chick kidneys within a shorter time compared with tracheal inoculation. This may be accounted for, at least in part, by differences in structure between their primary target organs. Intestinal tract, unlike trachea, offers a large superficial area for IBV infection with no ciliary movement to eliminate invading agents. Moreover, some absorptional functions or immune systems may transport much of the virus penetrating the intestinal wall into the systemic circulation. Kato *et al.* [5] demonstrated epithelial cells similar to M cells in the chicken intestine. Since M cells are known to play an important role in transporting viruses from the gastrointestinal tract to the circulatory system [15, 16], M cell-like cells may be involved in viral transportation from the gastrointestine to the kidneys in the chick.

Jones [4] suggested that although nephropathogenicity is a potential for most IBV strains, variants having strong nephropathogenicity are originated from non-nephropathogenic ones. In the present study, the incidence of nephrosis caused by the strain A-5968 increased more than 80% following *in vivo* passage in kidneys after cloacal inoculation. However, the incidence of nephrosis did not

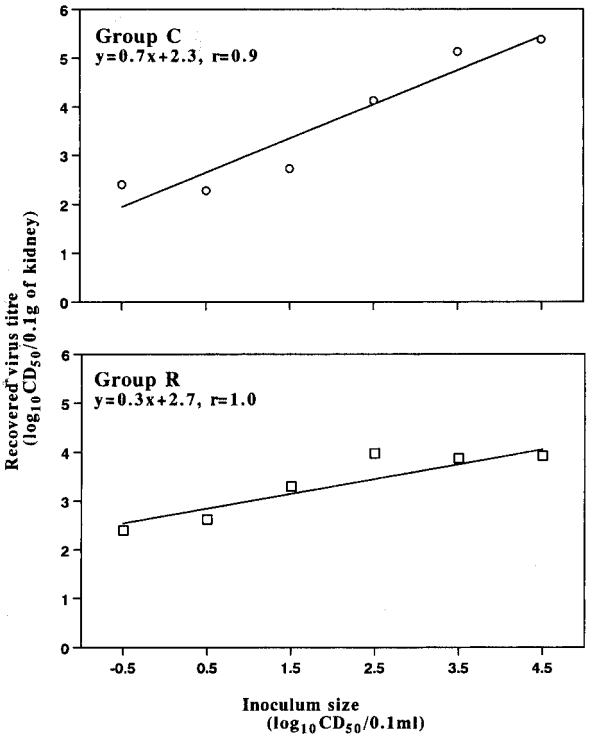

Fig. 4. Correlation between viral inoculum size and amount of virus recovery from kidneys of Groups C and R at 4 days PI.

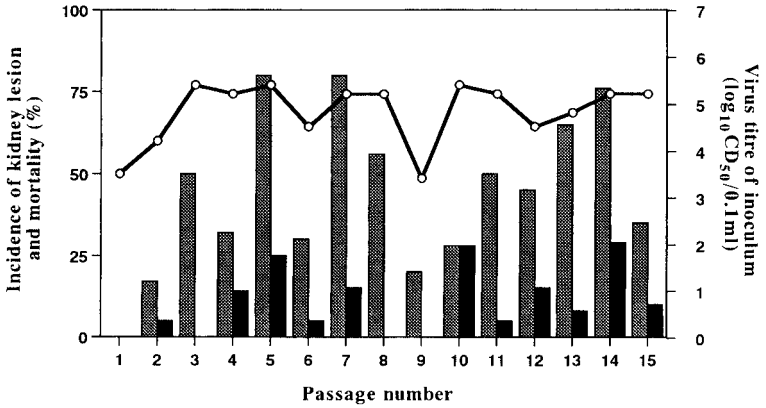

Fig. 5. Profile of incidence of kidney lesion (■), mortality (■) and virus titres in each inoculum (—○—) obtained during *in vivo* passage of strain A-5968 in kidneys using cloacal inoculation.

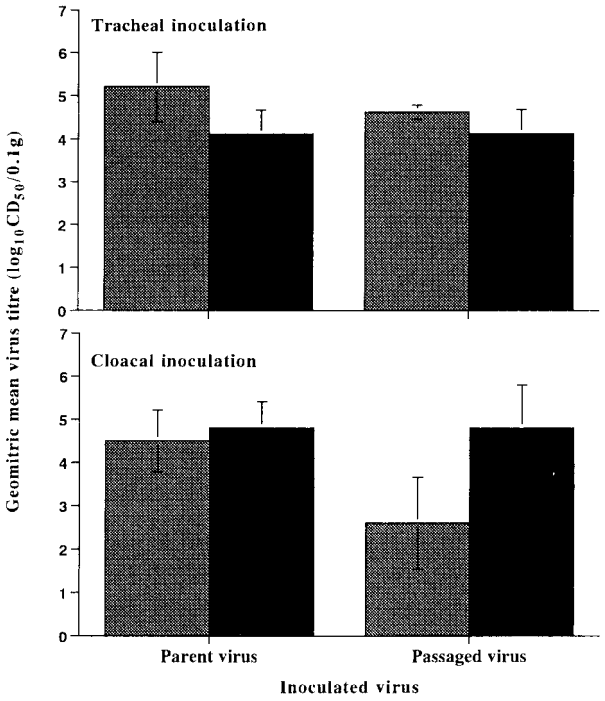

Fig. 6. Virus recovered from respiratory tracts (■) and kidneys (■) of chicks inoculated with parent or passaged virus intracloacally. * The value is significantly different from the value of parent virus cloacally inoculated.

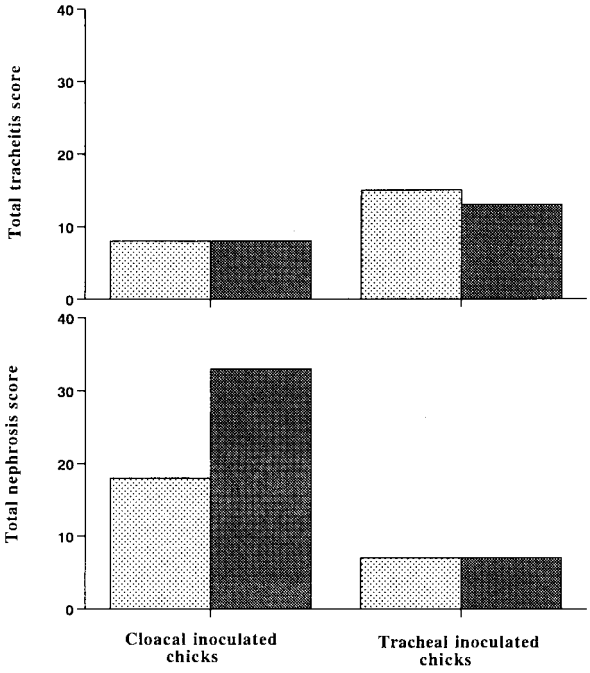

Fig. 7. Total scores of tracheitis and nephrosis caused by the parent virus (□) and passaged virus (■) in cloacal and tracheal inoculated chicks after histopathological examination.

Table 3. Cross serum neutralisation between parent and passaged IB viruses

Virus	Antiserum	
	Parent virus	Passaged virus
Parent virus	3.1[a]	2.4
Passaged virus	2.6	2.3

a) Serum neutralising antibody titre expressed as log_{10}.

always increased in proportion to the passage number. Most strains of IBV are able, to some extent, to grow in kidneys irrespectively of their organtropism, which may cause a difficulty in picking up only the subpopulation [9] having strong nephropathogenicity from this tissue.

SN antibody was produced more quickly following cloacal inoculation with the strain A-5968 than following tracheal inoculation, although chicks inoculated with the nephropathogenic IBV strain HS-91 via the cloaca showed similar SN antibody production to those inoculated via the trachea [13]. Toro *et al*. [12] reported that the cloacal route can be a vaccination route for live IBV vaccines. Chicks given vaccine via the cloaca may acquire earlier and stronger protection against virulent field infection than those given the vaccine via ordinary routes. It is concluded, therefore, that cloacal inoculation is useful for IB vaccination in the field.

The virus passaged 13 times via the cloaca caused more severe lesions in the kidneys of inoculated chicks than parent virus although there was no significant difference between both parent and passaged viruses in the amount of virus recovered from this organ. Thus the strain A-5968 is thought to acquire stronger nephrotropism with serially cloacal passages. Nevertheless it was surprised that the strain A-5968 did not necessarily acquired more strong nephropathogenesis with progression of cloacal passage in this investigation (Fig. 4). Incidence of kidney lesion and mortality of chicks inoculated cloacally fluctuated during serial passages. This incomprehensive phenomenon may be one of the characters of the strain A-5968. The result suggests that some factors which make IBV cause nephrosis, aside from the potential viral nephropathogenicity, are involved in the process of virus transportation from the cloaca to the kidneys. Furthermore, cloacal inoculation may also be a useful method to evaluate nephropathogenicity of IBV.

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