Effects of Bile and Gastrointestinal Secretions on the Infectivity of Newcastle Disease Virus

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Bile aspirated from chicken gall bladders was found to contain substances neutralizing Newcastle disease virus (NDV). Nonspecific factors were present in the bile, probably the bile acids, which caused a reduction in the infectivity of the virus. Specific anti-NDV activity was found in the bile of birds that had been vaccinated with a lentogenic strain, Ulster, and challenged with a velogenic, viscerotropic strain, California 1083. Immunoglobulins were also found in these secretions and demonstrated to include the immunoglobulin A class as well as immunoglobulin G. Variability in the neutralizing capacity of bile was found with two different plaque clones of strain 1083, indicating antigenic heterogeneity in the viral population. No difference was found between bile from uninfected birds and the bile from NDV-immune birds in their activities against influenza strain Turkey Ontario 7732, whereas activity existed against a non-viscerotropic strain of NDV, Texas GB. These findings suggest that the specific activity of the secretions was most probably due to the presence of secretory antibody. The importance of the antiviral substances present in the alimentary tract was discussed with respect to the protection of the chicken against the viscerotropic pathotype of NDV.

Newcastle disease is presently recognized as a major disease of poultry. The velogenic, viscerotropic form of the disease is characterized by the lesions of the gastrointestinal tract. If the agent establishes infection by the oral route, it seems reasonable that it must be relatively resistant to adverse conditions in the gut. Poliovirus, for example, can establish intestinal infection due to its resistance to extremely low pH values of gastric secretions (9). Other factors that may affect the infectivity of viruses are the presence of bile, urea, gastric mucin, and enzymes. Newcastle disease virus (NDV) is a paramyxovirus similar to the viruses which cause parainfluenza and mumps in man. Although NDV retains its infectivity over a large range of pH, from two to 10, for many hours (5), it is easily inactivated by treatment with lipid solvents and phospholipase C, which act on the lipid in the membrane. The bile acids are all powerful detergents and act as lipid emulsifiers facilitating the hydrolysis and absorption of dietary lipids in the gastrointestinal tract. This detergent property may enable the bile acids to play important roles in vivo in the inactivation of enveloped viruses exposed to the alimentary tract, but the effect of these acids on NDV has not been determined.

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secrections of the gastrointestinal tract may have on NDV infection.

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**MATERIALS AND METHODS**

**Experimental animals.** White Leghorn roosters about 5 to 6 months old were used in the experimental work. They were hatched and raised at the Reider Farm, University of Wisconsin, Madison, before their use. During the experiments, they were housed in a semi-isolation unit at Charmany Research Farm, University of Wisconsin, Madison. No anti-NDV antibody was detected in birds from the parent flock.

**Virus.** The strains of NDV used in this study were obtained as frozen stocks from the NDV repository at the University of Wisconsin. These strains included a lentogenic or avirulent strain, Ulster, and two velogenic or virulent strains, Texas GB and California 1083 (6). Early egg passages (fewer than five) were used. A virulent strain of influenza, Turkey Ontario 7732, was obtained as frozen stock for B. C. Easterday's use.

**Antisera.** Commercial preparations of antisera were employed for gel diffusion. These were rabbit anti-chicken IgG (Pentex Biochemical), rabbit anti-chicken IgG-7s fraction of antisera, rabbit anti-chicken globulin, and rabbit anti-chicken hemoglobin (Nutritional Biochemicals Corp.). A sample of rabbit anti-chicken IgA was kindly provided by John Bienenstock. This antiseraum reacts with the heavy chain of the immunoglobulin and a secretion specific component (2), which may be identical to a secretory piece.

**Production of anti-NDV sera and secretions.** The birds were given a vaccine of Ulster strain in drinking water (1 ml of Ulster stock virus to 1 liter of tap water, approximately $10^5$ 50% embryo infective dose (per ml). The Ulster strain was used because this strain can be regularly isolated from the intestinal tract soon after vaccination. The average consumption in the 4-h period the vaccine was available was about 100 ml or $10^7$ 50% embryo infective doses of the virus. The titer was confirmed by titration of the vaccine that remained. At 3-week intervals, thereafter, for a period of 9 weeks, the same group of roosters was exposed to a similar preparation of strain 1083, each time consuming about $10^5$ 50% embryo lethal doses on the average. The control birds received no exposure.

**Collection of serum and secretions.** The roosters were exsanguinated by cardiac puncture, and about 50 to 80 ml of blood was collected from each bird. The blood was allowed to clot, and the serum samples were separated, pooled, and heat inactivated at 56°C for 30 min.

The bile was collected from the gall bladders of the freshly killed birds with a 10-ml syringe and a 22-gauge 1.5 (about 3.81 cm) inch needle. Care was taken to avoid contamination with blood. The bile samples were immediately pooled.

Sections of the anterior duodenum about 30 cm long were removed. These were irrigated with approximately 25 ml of a warm solution containing 0.85% NaCl, 2% NaNO$_3$, and 0.006% trypsin inhibitor (soybean preparation; Sigma Chemical Co.). The washings were immediately pooled and kept cold in an ice bath.

The absence of blood contamination of secretion samples was established by testing in gel diffusion against rabbit anti-chicken hemoglobin. All samples were stored at 4°C overnight before further treatment.

**Preparation of samples.** The methods of Bienenstock et al. (2) for the preparation of immunoglobulins in secretions were modified for use in this experiment. The bile was centrifuged to remove insoluble material. An equal volume of cold saturated ammonium sulfate was added slowly to the bile to precipitate the immunoglobulins and other proteins. This mixture was then centrifuged at 5,000 × g for 30 min at 4°C to separate the precipitate. The supernatant was discarded and the precipitate was dissolved in 0.1 M tris(hydroxymethyl) aminomethane-hydrochloride and 0.2 M NaCl buffer, pH 8.0. To remove the salts, the preparations were dialyzed against the same buffer with 0.02% NaN$_3$ and 1.0 mM ethylenediaminetetraacetate added to prevent bacterial growth. The same procedure was used for the washings of the intestine and also for the serum. The preparations were stored at 4°C until used.

To clear the bile samples of low-molecular-weight components, they were filtered on Sephadex G-25 columns. The fractions which showed precipitin lines in double diffusion gels against anti-chicken gamma globulin were pooled. These were also the only fractions showing significant amounts of protein as measured by the biuret method of protein determination. The same buffer used previously was used in chromatography. After the fractions were pooled, they were concentrated by ultrafiltration to the original volumes of the samples. The quantities of protein in the final preparations of all the secretions were measured by the biuret method using horse gamma globulin as a standard (a chicken gamma globulin standard was unavailable). These protein measurements were intended only for use in adjusting the preparations relative to each other, not for the determination of the exact quantities.

**Virus titration in chicken embryos.** Decimal dilutions of the sample to be titrated were made in physiological saline (0.85% NaCl in twice distilled water), or in Earle balanced salt solution with 0.65% lactalbumin hydrolysate (Grand Island Biological Co.), 0.7% penicillin G, and 0.7% dihydrostreptomycin sulfate (Nutritional Biochemicals Corp.). Then, 0.1 ml of each dilution was injected into each of five 9- to 10-day embryonating eggs by the allantoic route (17). The inoculated eggs were incubated at 37°C and candled daily. Deaths were recorded, disregarding those occurring before 24 h.

**Virus titration by the modified egg-bit technique.** This technique (17) was used for titration of virus by placing 0.05 ml of each dilution of the virus preparation into each of five wells of a plastic tissue culture tray containing small bits of chorioallantoic membrane still attached to the shell and immersed in the medium of Fazeekas de St. Groth. They were then
incubated at 37 C for 36 h when the fluid from each well was removed and tested for hemagglutination.

**Hemagglutination.** For determination of hemagglutination titer of a sample, the standard method was employed (17).

**Plaque method.** Monolayers of chicken embryo fibroblasts were prepared by using Eagle minimum essential medium in Earle balanced salt solution (17). When the monolayers were confluent, they were washed with minimum essential medium and then inoculated with 0.05 ml or 0.1 ml of inoculum, depending on whether 30- or 60-mm tissue culture dishes were used.

**Gel filtration.** Gel chromatography was done by using Sephadex G-25, fine grade (Pharmacia Fine Chemicals Inc.). The gels was allowed to swell in the buffer previously described and then degassed in a vacuum flask overnight in the cold room (4 C). A 200-ml column was packed with the gel and equilibrated with buffer before samples were filtered. The fractions, 5 ml each, were collected by using an ISCO model UA ultraviolet analyzer (Instrumentation Specialties Co. Inc.).

**Ultrafiltration.** Bile preparations were concentrated by ultrafiltration in Amicon model 52 magnetically stirred cells equipped with PM-10 Diaflo membrane (Amicon Corp.). A positive pressure of 50 lb/inch² of N₂ was used.

**Immunodiffusion.** A modification of the Ouchterlony method (18) was used to perform double immunodiffusion in agar gel. The gel was composed of 1.9% agarose (agarose special grade, Schwarz/Mann) in 0.05 M sodium barbital hydrochloride buffer, pH 8.4, containing 0.001% trypan blue (Allied Chemical Corp.). Immunodiffusion was allowed to proceed at 4 C in a closed container with plenty of moisture, and the plates were observed daily for 2 to 4 days until the precipitin lines developed.

**RESULTS**

**Toxicity of bile and secretions.** Since embryonating eggs were used in many of the experiments with bile and secretions, it was first necessary to determine whether the preparations were toxic for the embryos and whether the preparations from infected birds contained any virus. Decimal dilutions of the bile samples from infected and uninfected birds were made in sterile saline. Five-tenths milliliter of each dilution was injected into each of three 7-day embryonating eggs via the yolk sac (17). Only one embryo death was observed in each group of three eggs injected with the undiluted bile samples. No deaths were observed in eggs injected with higher dilutions of the bile. Two-fold dilutions of the preparations of intestinal secretions were inoculated into 10-day embryonating eggs, 0.1 ml per egg, by the allantoic route. No embryo deaths occurred.

**Presence of immunoglobulins in secretions.** The presence of IgA was demonstrated in both bile and secretions from the duodenum in the gel diffusion test against rabbit anti-chicken IgA. This was confirmed by the fact that the identical component was found in serum and was not secretion specific. IgG was also observed in the secretions along with other non-immunoglobulin components which were present in serum. The protein contents, as measured by the biuret method, were 25 mg/ml for the concentrated bile samples and 4 mg/ml for dialyzed preparations of intestinal secretion.

**NDV specific antiviral activity of secretions.** To detect specific virus-neutralizing activity in the bile from NDV-immune birds, equal parts of fresh bile diluted 1:10 in saline were mixed with decimal dilutions of 10⁸3. These mixtures were incubated for 4 h at 37 C. One-tenth milliliter of each mixture was inoculated via the allantoic cavity of each of five 10-day embryos. The log₁₀ neutralization index was 6.5 for the bile from immune chickens, whereas the log neutralization index for the bile from controls was only 3.2.

The same procedure was employed to determine virus neutralization by the dialyzed ammonium sulfate preparations of the secretions and serum (Table 1). Relatively equivalent concentrations of protein were used in the experimental and control titrations for each secretion and for serum. To adjust the bile samples to equivalent concentrations, a scanning spectrum was run, using 10-fold dilutions of the concentrated bile samples. Peaks were observed at 325 and 800 nm, and the concentrations of bile were adjusted at these two values. A 10,000-fold neutralization was produced by bile preparations from immune roosters, whereas only a 10-fold neutralization was produced by the control bile. There was no significant neutralization by intestinal secretions.

The constant virus antibody dilution technique was employed to determine slight differences in the neutralizing capacity of the secretions. One hundred 50% embryonal lethal doses of 10⁸3 were incubated with twofold dilutions of each sample from immune and nonimmune roosters for 4 h at 37 C. One-tenth milliliter of each mixture was injected into the allantoic cavity of five 10-day embryonating eggs. Both the serum and bile preparations from immune birds completely neutralized the dose of virus (10 50% embryo lethal doses per egg) up to 20- and 40-fold dilutions, respectively. Neutralization 50% end points were greater than 20 for serum and 40 for bile from immune birds. As expected, the control bile preparation also neutralized the virus to some extent. Its neutralization 50% end point was 10. Neutralization by
the preparation from the intestines of immune birds was not significantly greater than preparations from controls. No neutralization was detected in the other samples tested.

Plaque neutralization in tissue culture was also performed by using the preparations of the secretions. One thousand plaque-forming units per milliliter of 1083, and twofold dilutions of the secretory preparations were mixed in equal volumes (Table 1). Difficulties were encountered with this method because at high concentrations the dialyzed preparations of the secretions destroyed the monolayers. In an attempt to eliminate this problem, 100 plaque-forming units/ml of 1083 were mixed with higher dilutions of the secretions (Table 1). Neutralization by both bile samples, the serum from NDV-immune birds, and possibly the intestinal secretions of the NDV-immune birds are indicated by these results.

In plaque neutralization studies with strain 1083, the virus used to challenge the birds, it was observed that almost invariably only red plaques were found after treatment with bile for infected birds. On the other hand, both red and clear plaques were found after treatment with bile from the control birds. Therefore, clones were made of the red and clear plaques and tested for neutralization by the bile samples. Table 2 presents the outcome of this experiment. These results are in agreement with the original observation that the bile from immune birds may be more effective against the clear plaque clone from strain 1083.

Another strain of NDV, Texas GB, was also tested for neutralization by the bile samples. Table 3 shows the results for Texas GB in embryos and egg bits, indicating that the bile from NDV-immune birds possesses activity against this strain of NDV as well as those used

<table>
<thead>
<tr>
<th>Determination</th>
<th>Control virus</th>
<th>Serum</th>
<th>Bile</th>
<th>Intestinal secretions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I+</td>
<td>N</td>
<td>I+</td>
</tr>
<tr>
<td><strong>Log ELD&lt;sub&gt;50&lt;/sub&gt;/ml</strong></td>
<td>8.6</td>
<td>Not done</td>
<td>Not done</td>
<td>4.6</td>
</tr>
<tr>
<td><strong>Log NI&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td></td>
<td>4.0±</td>
<td>1.0±</td>
<td>0.1±</td>
</tr>
<tr>
<td>50% Embryo neutralization&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&gt; 20</td>
<td>&lt;5</td>
<td>&gt; 40</td>
<td>10</td>
</tr>
<tr>
<td>50% Plaque neutralization&lt;sup&gt;f&lt;/sup&gt;</td>
<td>&gt; 64</td>
<td>&lt;2</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>50% Plaque neutralization&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&gt; 80</td>
<td>&lt;5</td>
<td>&gt; 80</td>
<td>20</td>
</tr>
</tbody>
</table>

<sup>a</sup> I, Immune; N, nonimmune.
<sup>b</sup> ELD<sub>50</sub>, 50% embryo lethal dose calculated by the Reed and Muench method (17).
<sup>c</sup> NI, Neutralization index.
<sup>d</sup> Assuming a value of 0.3 for the standard error of the samples (the maximum value calculated by the Spearman-Kärber method [4]), the difference between the neutralization by the bile samples was significant at P = 0.005 in the two-sample t test.
<sup>e</sup> Highest dilution of sample which caused 50% neutralization of 10 ELD<sub>50</sub> of California 1083.
<sup>f</sup> Highest dilution of sample which caused 50% reduction of 100 plaque-forming units of California 1083.
<sup>g</sup> Highest dilution of sample which caused 50% reduction of 10 plaque-forming units of California 1083.

<table>
<thead>
<tr>
<th>Determination</th>
<th>Control virus</th>
<th>Original 1083</th>
<th>Control virus</th>
<th>Large clear plaque</th>
<th>Control virus</th>
<th>Large red plaque</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bile</td>
<td>Bile</td>
<td>Bile</td>
<td>Bile</td>
<td>Bile</td>
</tr>
<tr>
<td><strong>Log ELD&lt;sub&gt;50&lt;/sub&gt;/ml</strong></td>
<td>8.3 ± 0.3</td>
<td>7.0 ± 0.3</td>
<td>8.2 ± 0.2</td>
<td>7.3 ± 0.2</td>
<td>5.5 ± 0.3</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td><strong>Log NI&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td>1.3 ± 0.2</td>
<td>0.1 ± 0.3</td>
<td>1.8 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> I, Immune; N, nonimmune.
<sup>b</sup> ELD<sub>50</sub>, 50% embryo lethal dose; calculated by the Spearman-Kärber method (4).
<sup>c</sup> NI, Neutralization index.
<sup>d</sup> The difference between the neutralization by the bile samples of the red plaque clone and the clear plaque clone was significant when using the two-sample t test at P = 0.005.
for vaccination and challenge.

Finally, to determine the specificity of the antiviral action of the bile, the bile samples were tested against a strain of influenza, Turkey Ontario 7732. This strain was chosen because its biological properties are similar to those of velogenic strains of NDV, whereas there is no evidence for cross-reaction in the neutralization of NDV and influenza. The virus treated with bile from immune birds was reduced in titer by 1.2 logs, whereas the virus treated with the bile preparation from control birds was reduced by 1.0 log (Table 4). No significant difference was found between these values.

**DISCUSSION**

The present study has shown that the bile from uninfected chickens definitely inactivates NDV. A 10^4-fold inactivation of strain 1083 by fresh bile was observed. Dialyzed ammonium sulfate precipitates of bile inactivated the virus 10-fold. Inactivation is presumably due to the disruptive detergent action of the bile acids on the lipid bilayer of the viral membrane. This effect may be sufficient to protect the bird when the virus titer in consumed material is very low. However, situations may arise when the virus titer is as high as 10^8 or 10^6 50% embryo lethal doses and infection could then occur. It is also possible that the virus may be able to avoid the antiviral effects of bile through protection by other organic substances introduced into the alimentary tract.

Chickens infected with NDV produce local secretory immunoglobulins, probably IgA, which are detectable in bile and intestinal secretions. Fresh bile and dialyzed ammonium sulfate precipitates of bile for NDV-immunized birds neutralized NDV in three different assay systems. Fresh bile for immune birds produced over a 6-log reduction in the infectivity of 1083, whereas bile from control birds produced only a 3-log inactivation. A 3-log difference in neutralization by the two types of bile was also observed after the bile samples were processed. In addition, neutralization end points for bile from immune birds assayed by the constant virus-antibody dilution technique were significantly greater than end points for bile from nonimmune chickens. No significant neutralization was produced by the intestinal secretions from immune birds relative to those from control birds. The low protein content in the intestinal secretions may account for this failure to observe neutralization. Assay of neutralization activity by plaque reduction employing bile and intestinal secretion from immune and nonimmune birds further confirmed these results.

The specific nature of the observed neutralization is further supported by the demonstration that the bile from NDV-immune birds also neutralized another strain of NDV, Texas GB, but there was no significant difference between the bile from immune and nonimmune birds when tested against influenza strain Turkey Ontario 7732. This indicated that the stronger antiviral action of the bile from immune birds is due to specific antibody to NDV and not due to another less specific substance such as interferon.

### Table 3. Neutralization in chicken embryos and egg bits by bile with the constant sample-virus dilution technique with the non-viscerotropic strain Texas GB

<table>
<thead>
<tr>
<th>Determination</th>
<th>Chicken embryos</th>
<th>Egg bits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control virus</td>
<td>Bile</td>
<td>Bile</td>
</tr>
<tr>
<td></td>
<td>I (^a)</td>
<td>N (^f)</td>
</tr>
<tr>
<td>Log ELD(_{50})/ml(^c)</td>
<td>9.2</td>
<td>6.6</td>
</tr>
<tr>
<td>Log NI(^c)</td>
<td>2.6</td>
<td>0.8(^f)</td>
</tr>
</tbody>
</table>

\(^a\) I, Immune; N, nonimmune.

\(^b\) ELD\(_{50}\), 50% embryo lethal dose; calculated by the Reed and Muench method (17).

\(^c\) NI, Neutralization index.

\(^f\) The differences in neutralization by the bile samples in both techniques were found to be significant at \( P = 0.005 \) by the two-sample t test (values of 0.3 were assumed for the standard errors).

### Table 4. Neutralization in chicken embryos of influenza-Turkey Ontario 7732 by bile with the constant sample-virus dilution technique

<table>
<thead>
<tr>
<th>Determination</th>
<th>Control virus</th>
<th>Bile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I (^h)</td>
<td>N (^f)</td>
</tr>
<tr>
<td>Log ELD(_{50})/ml(^c)</td>
<td>5.1 ± 0.3</td>
<td>3.9 ± 0.3(^f)</td>
</tr>
<tr>
<td>Log NI(^c)</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>

\(^a\) I, Immune; N, nonimmune.

\(^b\) ELD\(_{50}\), 50% embryo lethal dose.

\(^c\) The differences between the neutralization by the bile samples were not significant using the two-sample t test at \( P > 0.05 \).

\(^f\) NI, Neutralization index.
The presence of immunoglobulins in secretions shown in this study is in agreement with previously published results (1–3, 13). The bile contained IgA and IgG as well as other non-immunoglobulin components which were found in serum. A bile-specific component, possibly secretory piece, was also found. IgA appeared to be the major immunoglobulin the upper intestine as well; IgG was usually seen only after concentration of samples.

Interestingly, neutralizing capacity of the secretions shows varied specificity for the clones of two plaque types of 1083. The clear-plaque type was much more sensitive to neutralization by the bile from immune birds than the red-plaque clone. The difference may be due to antigenic variability of the viscerotropic strain and may be another reason why this type of virus may be able to evade the immune system of birds which were previously vaccinated with a strain such as B1.

The presence of local immunity in the intestinal tract in Newcastle disease infections is in agreement with the work of Kono et al. (12), who found anti-NDV antibody in extracts of the feces of chickens immunized orally. In fact, it would be surprising if there were not some degree of local immunity in viscerotropic NDV infections, since local immunity plays such an important role in infections such as polio and transmissible gastroenteritis. It is known by the studies of Kohn and Ebert (11) that NDV can infect the intestinal epithelium of isolated intestinal loops, subsequently spreading to other sites. The route of infection has a pronounced effect on the dosage required for infection, the oral route requiring much larger quantities of virus than the respiratory route (10). The reason for the larger dose required for oral infection is presumed to be the detrimental action of substances in the alimentary tract on the virus.

The antibody in the intestinal tract may not attain the high neutralizing levels shown by serum antibody, but when viewed with respect to the other antiviral substances present in such an environment, it may have an amplifying effect on the protection of the bird. The findings here are consistent with the conclusion of Parry and Aitken (16) that the local immune response may be most effective against repeated low levels of infecting virus. Certainly, the high levels of virus which can reach the respiratory surfaces would not reach the surface of the intestinal epithelium, even in the infection of a viscerotropic virus, such as 1083. Therefore, the combined mechanisms of the bile, enzymes, and other substances present in the gastrointestinal tract, plus the antibody produced locally, may be sufficient to prevent infection by that route when neither the nonspecific substances nor antibody alone could provide protection.

ACKNOWLEDGMENTS

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LITERATURE CITED