Factors Influencing the Appearance of Antibody in Tracheal Washes and Serum of Young Chickens After Exposure to Newcastle Disease Virus

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The development of plaque-neutralizing antibody in tracheal washes and hemagglutination inhibition antibody in serum was followed after intratracheal and intranasal inoculation of 1-, 14-, or 28-day-old chicks with a lentogenic strain of Newcastle disease virus (NDV). Serum antibody could be detected between 7 and 10 days after intratracheal and intranasal vaccination in birds either with or without maternal antibody to NDV. However, among the 1-day-old group only birds without maternal antibody showed an antibody response after intramuscular inoculation. All birds possessing either actively or passively acquired serum antibody showed a sharp rise and subsequent decline of anti-NDV activity in tracheal washes between 4 and 10 days after intratracheal or intranasal vaccination. Using radiolabeled chicken immunoglobulin injected intravenously as a tracer, it was shown that this initial peak of anti-NDV activity in tracheal washes could be accounted for by enhanced transudation of serum antibody. The transudation of serum antibody coincided with the course of viral pathology observed in the tracheae of infected birds. Neutralizing antibody in tracheal washes beyond 10 to 14 days postvaccination was, most likely, produced locally, in the respiratory tract.

A body of both indirect and direct evidence has been accumulated that supports the existence of a local immune system in the respiratory tract of the chicken. Protection from reinfection by Newcastle disease virus (NDV) (4, 6, 21) or infectious bronchitis virus (19) has been best correlated with prior exposure of the respiratory epithelium to the infectious agent rather than with levels of serum antibody. Studies involving the use of tracheal organ cultures have shown that tracheal explants obtained from birds immunized by respiratory routes produced neutralizing antibody (13, 14, 17, 18) and resisted reinfection better than explants obtained from birds immunized by intramuscular (i.m.) or intraperitoneal routes (13, 30). Furthermore, specific neutralizing activity has been detected in secretions of the respiratory tract after local exposure to infectious bronchitis virus (19) or NDV (1, 23, 27, 32, 33). Antibody of the immunoglobulin G (IgG) (32, 33) or IgA (26, 27) classes has been implicated as a mediator of this activity.

Few attempts have been made to follow the appearances of antibody in respiratory secre-

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tions during the period after antigenic exposure, particularly in newly hatched chicks. The difficulties of collecting samples without serum contamination and obtaining enough fluid to accurately measure the low levels of activity present have discouraged such investigations. However, such a study would be of particular interest in poultry because immunization is more convenient to implement just after hatching and because of the high susceptibility of young birds to infection and death by NDV.

The present study was undertaken to determine what effect age, route of vaccination, and maternally acquired antibody have on the appearance of antibody in respiratory secretions after vaccination of chicks within the first few weeks after hatching. Although the development of serum antibody has been shown to be adversely affected by the presence of maternal antibody, the effects of these factors on the development of antibody in the respiratory tract have not been explored. Also, evidence suggesting that serum antibody may contribute to the neutralization of virus in the respiratory tract (6) has not been substantiated and may have implications concerning the role of maternal antibody in protecting the respiratory tract from infection.
MATERIALS AND METHODS

Animals. One-day-old White Leghorn chickens possessing maternally derived antibody to NDV were obtained from a local commercial hatchery. White Leghorn chickens free of maternal antibody to NDV were hatched in isolation, from eggs purchased from Specific Pathogen Free Avian Supply (SPAFAS), Norwich, Conn. During these experiments, birds were maintained in modified Horsfall-Bauer units supplied with filtered air and under positive pressure.

Vaccination. A B-1 strain of NDV (Sterwin Laboratories, Inc., Millaboro, Del.) having a titer of 10³ mean effective lethal doses per ml was used throughout these experiments, with the exception of experiment 1, when a β-propiolactone-inactivated La Sota strain of NDV was given i.m. In the latter case, 0.1 ml of killed vaccine was injected in each breast and thigh muscle. Aerosol exposure of the chickens was conducted in a room (20 by 15 feet (ca. 6.1 by 4.6 m)) by aerosolizing the contents of a 1,000-dose vial of B-1 vaccine diluted in 125 ml of deionized water containing 2% nonfat dried milk. The birds were left in the room for 30 min and returned to the isolation units. B-1 vaccine diluted 1:10 in sterile phosphate-buffered saline (PBS) was also administered locally by injecting 0.1 ml in each nostril and 0.4 ml into the trachea. Intramuscular vaccination was accomplished by injecting 0.1 ml of the B-1 vaccine stock in each breast.

Collection of samples. Before obtaining samples the chicken was killed with an overdose of sodium pentobarbital given intraperitoneally. The thoracic cavity was opened, and the bird was exsanguinated from the heart. Serum was extracted from the clotted blood by centrifugation and then frozen at −20°C. The trachea was exposed by making an incision along the ventral side of the neck with blunt-end scissors; clamps were placed at both ends, and the trachea was removed by transecting it just outside each clamp. During the excision the head and thorax were elevated to prevent any fluids that accumulated from entering the bronchi. The outside of the trachea was blotted with cotton gauze; then 4 ml of cold PBS was slowly flushed through the lumen of the trachea into a glass tube. Samples were held on ice and subsequently lyophilized. The lyophilized samples were reconstituted with 1.0 ml of sterile distilled water before analysis. Portions of each trachea were processed for histological examination by the method of Sainte-Marie (29) and stained with hematoxylin and eosin.

Analysis of serum and tracheal wash fluids. Protein concentrations in tracheal wash samples were determined by the method of Lowry et al. (22) with bovine serum, fraction V, as a standard. Serum samples were analyzed for anti-NDV antibody by the hemagglutination inhibition test as described elsewhere (25), using 10 U of antigen per well. The plaque neutralization test was performed according to a standard procedure (10). Three-day-old monolayers of embryonic chicken kidney cells were inoculated with twofold dilutions of tracheal wash samples preincubated with a standard dilution of indicator virus (Kansas-Manhattan strain of NDV). Plaques were visualized after 72 h of incubation by addition of neutral red, and the mean number of plaques on duplicate plates for each dilution was determined. The titer was determined as the highest dilution of sample that yielded between 45 and 55% as many plaques as found on plates inoculated with a mixture of indicator virus and negative serum. Selected tracheal wash samples were assayed for interferon activity by the inhibition of plaque formation by vesicular stomatitis virus as described elsewhere (15).

Preparation of anti-IgG immunoabsorbent. Antiserum against chicken IgG was prepared in goats. A 14% Na₂SO₄ (wt/vol) precipitate of serum from 1-day-old chickens was passed over a column of Sephadex G-200. The second peak to emerge from the column was used for immunization. A goat was inoculated with 30 mg of protein in complete Freund adjuvant. Booster inoculations were given at 4-week intervals with 10 to 20 mg of IgG in incomplete Freund adjuvant. Antibody against IgG was removed from serum by adsorption on an immunoabsorbent of the chicken IgG prepared above coupled to Sepharose 4-B (24). The antibody that eluted from the column with 3 M NaSCN in 0.15 M tris(hydroxymethyl)aminoethanehydrochloride, pH 7.4, was passed over bile IgA and gammaglobulinemic plasma (a gift from D. Y. E. Perey) immunoabsorbents to remove non-class-specific activity. IgA was extracted from bile by passage over Sephadex G-75 to remove low-molecular-weight salts, followed by an agarose A-1.5 column (Bio-Rad Laboratories, Richmond, Calif.). The IgA eluted from the agarose column as a single uniform peak of protein. After absorption the antiserum was shown to be specific for IgG by double diffusion in gel and immunoelectrophoresis with serum proteins. The goat anti-IgG globulins were coupled to Sepharose 4-B by the method of March et al. (24) and used for adsorption of radiolabeled IgG as described below.

Iodination of IgG. Globulin isolated from serum of 1-day-old chickens as described above was shown to contain only IgG by immunoelectrophoresis with rabbit anti-whole chick serum. This IgG preparation was coupled with 125I by using chloramine-T as described elsewhere (16). Unbound 125I was removed by extensive dialysis until 93% of the activity in the labeled preparation could be shown to be precipitable with trichloroacetic acid.

Experimental design. (i) Experiment 1. One-day-old White Leghorn chickens with moderate levels of maternal antibody to NDV (geometric mean titer, 40) were divided into two groups of 25 birds each; one group was aerosol vaccinated with NDV-B-1, and the other group was vaccinated i.m. with inactivated NDV-La Sota; a third group of 20 birds remained uninoculated. Five birds from each vaccination group and three birds from the control group were sacrificed just before vaccination and at 4, 7, 10, 14, and 21 days after vaccination for acquisition of samples.

(ii) Experiment 2. Two groups of 25 1-day-old chickens, one with and the other without maternal antibody to NDV, were inoculated intratracheally (i.t.) and intranasally (i.n.) with NDV-B-1 as described previously; 20 birds from each source remained unexposed. Each group was maintained in separate isolation units. Five birds from each vaccination group and three birds from each control group were sacrificed before vaccination and at 4, 7, 10, 14, and 21 days after vaccination.
postvaccination, and samples were taken as previously
described.

(iii) Experiment 3. Chickens hatched from
SPAFAS eggs were maintained in a modified Horsfall-
Bauer unit until used; on days 14 and 28 after hatching,
70 birds were removed from isolation; on each date,
25 birds were injected i.m. with live NDV-B-1, and
another 25 birds were inoculated i.t. with the same
stock of virus diluted 1:10 in sterile PBS. A third
group of 20 birds remained unexposed. Each group
was maintained in a separate isolation unit. At 4, 7,
10, 21, and 28 days after vaccination, 5 birds from
each vaccinated group and 3 from the control group
were sacrificed, and samples were collected. Samples
were also collected from 5 of the birds before vacci-
nation.

(iv) Experiment 4. Fifty-five 1-day-old White Leg-
horn chickens were maintained in a modified Horsfall-
Bauer unit; at 14 days of age each bird was injected
intravenously with 0.5 ml of PBS containing 1 mg of
131I-labeled chicken IgG per ml. Four hours later 20
birds were inoculated i.m. with live NDV-B-1; another
20 birds were inoculated i.t. and i.m. with the same
virus stock as described before; 15 birds remained
unexposed. Each group of birds was maintained in a
separate isolation unit. At 2, 4, 7, 10, and 14 days
postinoculation, 4 birds from each vaccination group
and 3 from the control group were killed, and tracheal
wash and serum samples were obtained as described
previously. Hemolyzed sera or blood-contaminated
tracheal washes (although rare) were discarded; dupli-
cate 0.2-ml samples of both serum and tracheal washes
were placed in 7-ml plastic miniscintillation
vials and suspended in 4 ml of scintillation cocktail.
The activity in each sample was counted in a Packard
Tri-Carb liquid scintillation spectrometer. After sub-
traction of background activity, the counts of duplicate
samples were averaged, and the data were plotted as
the mean ratio of the counts per minute for tracheal
wash sample to the counts per minute of the corre-
sponding serum sample for birds on a given day.

RESULTS

Analysis of sampling technique. In experi-
ment 3 a mean volume of 3.8 ± 0.08 ml was
recovered after injecting 4.0 ml of PBS into each
trachea. This represents a standard sampling
error of 2.2%. A minimum dilution factor of 1/20
was estimated for initial washings, and a factor
of 1/5 was estimated for the reconstituted lyophi-
лизed samples. However, these factors were not
taken into account in the representation of the
antibody titers. The protein content of tracheal
washes during the 3 weeks after vaccination of
4-week-old birds (experiment 3) is given in Fig.
1. Each point represents the mean of eight sam-
ples. Although the protein levels varied widely
between birds of the same treatment group sam-
ped on the same day, a marked elevation of
protein was detected among birds vaccinated
i.t. At 4 days after exposure these birds had a
mean protein level about five times that of the
i.m.-vaccinated or control group. The mean pro-
tein levels of the i.m.-vaccinated group rose
slightly 7 to 10 days postinoculation. The protein
levels of tracheal washes obtained from both
vaccination groups returned to the level of the
control group by 14 days postexposure. In gen-
eral, during the course of these experiments
there was no neutralizing activity detected in
tracheal washes either against NDV in uninocu-
lated birds or against vesicular stomatitis virus
samples with or without neutralizing activity
against NDV.

Experiment 1: Development of antibody
in serum and tracheal washes following i.m.
or aerosol vaccination of 1-day-old
birds. Both nonvaccinated birds and i.m.-vac-
cinated birds showed similar declines in matern-
ally derived serum antibody, reaching unde-
tectable levels by 3 weeks of age (Fig. 2). No
neutralizing activity was detected in tracheal
wash samples of either group. The serum anti-
body levels of the aerosol-vaccinated group first
declined, then rose on day 10 to a level three dilutions above that of the other groups, and remained at a detectable level 3 weeks after exposure. The most noticeable difference between routes of exposure was in the high level of specific neutralizing activity in tracheal washes 7 days after aerosol vaccination. The level of activity subsequently declined below a detectable level 21 days after exposure. The presence of live vaccine virus in tracheal wash samples collected 4 days after aerosol vaccination complicated detection of anti-NDV antibody in this and subsequent experiments.

Experiment 2: Effect of maternal antibody on development of serum and tracheal wash antibody after i.t. and i.n. exposure to NDV-B-1. Serum antibody titers of birds with or without maternally derived antibody to NDV reached about the same maximum level about 10 days after vaccination (Fig. 3). The onset of antibody production, which was apparently masked by maternal antibody, could be detected sooner, i.e., 7 days after exposure, in birds lacking maternal antibody. The appearance of antibody in tracheal washes did not appear to be affected by the presence of maternal antibody. In both groups tracheal wash antibody was not detected until after active antibody production was evident in serum. Maximum levels of neutralizing activity in tracheal washes were reached after 10 days postexposure.

Experiment 3: Influence of route of exposure to NDV on the development of serum and tracheal wash antibody in 2- and 4-week-old birds. In the absence of maternal antibody, virus administered either i.m. or locally to the respiratory tracts induced similar antibody responses in both 2- and 4-week-old birds (Fig. 4 and 5). The serum antibody titers of the 4-week-old birds appeared to continue to increase 3 weeks after exposure, while, at the same time, the level of activity was on the decline in 2-week-old birds. The most obvious difference in the routes of exposure was reflected in the appearance of neutralizing activity in tracheal washes. Local application of virus to the respiratory tract resulted in a sharp increase in tracheal wash antibody in 2- and 4-week-old birds. At both ages a maximum level was detected at 7 days after exposure followed by a rapid decline in activity. In contrast to the steady decrease in 2-week-old birds, the levels of tracheal wash antibody in 4-week-old birds began to rise 3 weeks after exposure. I.m. vaccination resulted in an erratic appearance of antibody in tracheal washes. Maximum levels of antibody, at least three dilutions below those attained after local vaccination, were reached 7 and 21 days after i.m. inoculation of 2- and 4-week-old birds, respectively. Clearly, local application of virus had produced a higher, although transient, level of antibody in tracheal washes, a level that was sustained in 4-week-old birds.

Experiment 4. Contribution of serum antibody to the contents of tracheal washes after i.m. or i.t. vaccination. Histological examination of tracheae from chickens vaccinated i.t. and i.m. revealed, in general, that maximum destruction of the ciliated epithelium occurred between 4 and 7 days after exposure to the virus. By day 7 the epithelium had partially regenerated, and 14 days after exposure the tracheal epithelium appeared normal. The tracheal epi-
Intratracheally Vaccinated Intramuscularly Vaccinated

**FIG. 4.** Development of local and systemic antibody in chickens after vaccination with NDV at 2 weeks of age (experiment 3).

Intratracheally Vaccinated Intramuscularly Vaccinated

**FIG. 5.** Development of local and systemic antibody in chickens after vaccination with NDV at 4 weeks of age (experiment 3).

The luminal fluid of i.m.-inoculated birds as well as that of unexposed controls were observed to be undamaged during the experiments. These observations suggested that serum antibody may leak into the respiratory tract during the height of a local NDV infection.

To investigate this possibility, radiolabeled IgG injected intravenously served as a tracer for transduced serum antibody in tracheal washes of i.t.- or i.m.-vaccinated birds. The proportion of the activity found in tracheal washes relative to that found in serum of given birds after exposure to NDV vaccine is shown in Fig. 6. Two days after vaccination the amount of activity in tracheal washings of i.t.- or i.m.-inoculated birds was, essentially, at the same level as that of the unexposed birds. By day 4 there was a sharp rise in tracheal wash activity in the i.t.-vaccinated birds, which represented over 25% of the activity in serum. This level of relative activity decreased slightly on days 7 and 10, reaching the level of the controls around day 14. The relative activity in tracheal washes of i.m.-vaccinated birds was at the level of the control group in all samples except those collected on day 4. The mean activity of tracheal washes of the 4-day samples represented about 12% of the activity found in serum.

The tracheal wash samples obtained 4 days after i.t. vaccination were tested to determine the amount of activity that remained associated with the immunoglobulin molecule after passage through the chicken. Samples were absorbed by goat anti-chicken γ-chain globulin bound to Sepharose 4-B. After thorough washing, a mean of 93% of the activity of the washes remained bound to the immunosorbent. A control immunosorbent consisting of the same amount of normal globulin bound to Sepharose absorbed only 25% of the activity present in the same samples. Therefore, it was concluded that between 60 and 90% of the activity detected in tracheal washes remained associated with the immunoglobulin molecule.

**DISCUSSION**

The techniques of collecting samples from the respiratory tract are crucial to a meaningful analysis of the local antibody response. Ideally, quantitative, reproducible samples should be obtained in such a way as to avoid serum contamination resulting from manipulation of the animal during sampling. The procedure described herein adequately meets these criteria. Also, techniques that rely on standardization of antibody titers with respect to the protein content of samples may give an erroneous representation of the antibody response in some secretions, particularly when cytolytic or infectious agents are used to elicit a local immune response. As the present data show, the protein content under these circumstances is a reflection of the infectious process and cannot be a reliable reference standard between birds or sampling times.

The results of these experiments demonstrate that active antibody production in young birds becomes evident at about 7 days postvaccination. Similar results have been obtained following i.t. (11) or i.n. (6) vaccination of 1-day-old birds. These results also support the contention

**FIG. 6.** Transudation of radiolabeled chicken IgG from serum into tracheal secretions after vaccination with NDV (experiment 4).
that local application of virus to the respiratory tract is superior to i.m. inoculation for eliciting a serum antibody response in young passively immune birds (7). i.m. administration of inactivated La Sota vaccine virus, which was shown capable of eliciting an antibody response in non-immune birds, was ineffective in birds that had moderate levels of maternal antibody. On the other hand, live virus administered i.t. or i.n. to 1-day-old birds elicited an antibody response that was detectable 7 days later. It is reasonable to assume that virus deposited in the respiratory tract was able to evade neutralization by serum antibody and to infect the epithelium. The virus was then able to persist in the young birds at a time when immunocytes capable of responding to antigenic stimulation were increasing in number (31). By 6 to 7 days of age both the decline in maternal antibody and the increase in antigenic mass permitted stimulation of active antibody production. i.m.-administered virus was, presumably, neutralized before it could establish an infection and stimulate antibody production.

Although local application of virus resulted in a sudden, transient appearance of antibody in the trachea, the source of the antibody was not apparent. It was anticipated that if serum antibody entered the trachea as a result of the viral infection it would be detected first in birds that possessed maternal antibody and would be detected in birds without passive antibody only after antibody synthesis commenced. However, neutralizing antibody was uniformly detected on day 7 after exposure in birds both with and without maternal antibody to NDV. As pointed out previously, detection of antibody in tracheal washes collected 4 days after i.t. vaccination was complicated by the presence of vaccine virus, which, most likely, absorbed any antibody entering these secretions. Consequently, antibody could only be detected when in excess of that which could be absorbed by viral antigen. Although maternal antibody may have entered the trachea before 7 days postvaccination, its detection was blocked by the presence of virus, and passive transudation of serum antibody could not be distinguished from local synthesis as the source of antibody in the trachea.

In 2- and 4-week-old birds, active antibody production, as indicated by the appearance of antibody in serum, was first evident 7 days after vaccination. In these older birds, in which both maternal antibody and immunological incompetence were no longer limiting factors, both routes of inoculation produced similar serum antibody responses. Again, it was not clear whether the peak of neutralizing antibody in tracheal washes was the result of local production or enhanced transudation of serum antibody, since its detection coincided with the appearance of antibody in serum.

At least two lines of evidence suggest that the majority of this initial peak of antibody activity in tracheal washes is derived from serum. The histological changes observed in the trachea following i.t. vaccination with a lentogenic strain of NDV coincide with previous reports of the course of infection in both the upper (3) and the lower (5) respiratory tracts. This disruption of the epithelial barrier between 4 and 8 days after local application of NDV vaccine coincided with the timing of the peak of neutralizing antibody activity and radiolabeled IgG, which appear in tracheal washes after local application of NDV vaccine. Also, it is not unreasonable to attribute the slight rise in neutralizing activity and radioactivity after i.m. inoculation to an increase in capillary permeability in the trachea induced by the virus. This interpretation is supported by the finding by Beard and Easterday (4) that live virus could be isolated from tracheae 4 days after i.m. inoculation of B-1 NDV without any gross histological damage to the trachea.

Passive transudation of serum proteins into the secretions of the respiratory tract and oral cavity has been observed in other animals as well as man after either infection (2, 9, 12) or mechanical irritation (8).

In chickens, Leslie et al. (20) have shown that in normal birds low levels of radioactivity can be detected in upper alimentary tract secretions after intravenous administration of radiolabeled IgG. Also, in a recent report (1) the amount of serum-derived antibody against bovine serum albumin or sheep erythrocytes in lachrymal and salivary secretions was found to be significantly higher 7 days after ocular application of NDV vaccine than that in nonvaccinated birds. However, in the same and in a previous report (27) it was concluded that the majority of the antibody appearing in these secretions as well as in tracheal washings within 10 days after local vaccination was of local origin, whereas serum antibody appeared to make a relatively more significant contribution around 18 days after vaccination. The conclusions concerning the minor role of serum antibody during the acute phase of the infection were supported by the failure of infectious bronchitis virus to induce marked transudation of serum anti-NDV antibody into the respiratory tract after ocular application (1).

As the authors expressed and our observations indicate, this finding may not be representative of the effect that NDV has on the tracheal epithelium and transudation of serum proteins. Also, the ocular route of vaccination may not present enough virus to the tracheal epithelium to cause a pronounced leakage of serum anti-
body, as was observed in this report.

The findings reported herein imply that serum antibody can neutralize locally applied vaccine virus in the respiratory tract before the virus ever enters the circulation. Consequently, in young chicks maternal antibody may neutralize low doses of live vaccine virus given by respiratory routes. This conclusion is supported by an earlier observation of Beaudette and Bivins (6). They found that after administration of low levels of live NDV vaccine to passively immune chicks the virus produced only a transient infection, and only large doses of virus proved effective in establishing an infection and eliciting a serum antibody response. In nonimmune birds even the lowest dose of virus established a local infection and induced an immune response. Therefore, although the respiratory tract may provide the vaccine virus a sanctuary from maternal antibody in newborn chicks, it is a temporary one. Future studies of the efficacy of local vaccination procedures should take into account the effect of maternal antibody, which appears in secretions when live virus is used.

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LITERATURE CITED


