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Local Antibody Response in Chickens: Analysis of Antibody Synthesis to Newcastle Disease Virus by Solid-Phase Radioimmunoassay and Immunofluorescence with Class-Specific Antibody for Chicken Immunoglobulins

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The antibody response to Newcastle disease virus was monitored in the sera and salivas of adult chickens immunized by two methods: (i) combined intratracheal-intranasal vaccination followed by intratracheal revaccination or (ii) intramuscular vaccination followed by intratracheal revaccination. By solid-phase radioimmunoassay, only immunoglobulin G (IgG) and IgA antibodies to Newcastle disease virus were detected in the salivas, whereas IgA and IgM antibodies were present in egg whites. The first method produced the highest antibody levels in both serum and saliva and, in addition, prevented detectable virus multiplication in the respiratory tracts upon revaccination 4 weeks later. Plasma cells of all three classes were distributed throughout the tissues lining the oral cavities. The highest densities of plasma cells were in the Harderian glands; IgG was the predominant class, whereas IgA and IgM plasma cells were present in almost equal but lower numbers. The Harderian plasma cells were the most likely source of the antibody found in saliva.

The importance of locally produced antibodies in the defense of mucosal surfaces against viral infections has been well documented in humans and animals (12, 24, 26, 27). Several reports have demonstrated the importance of such antibodies in the respiratory tracts of chickens subjected to local application of antigen (2, 13, 23, 30, 35, 36). In particular, since the primary site of infection of Newcastle disease virus (NDV) is the respiratory tract (17, 18), emphasis has been placed on the local application of NDV vaccines by eyedrop and aerosol methods (2, 3, 30). However, analysis of the local antibody response has been frustrated by the paucity of antibody produced and the difficulty of quantitatively collecting samples. Consequently, despite indications that under given conditions local immunity in the respiratory tract may develop in the absence of serum antibody, the primary indicator of the efficacy of vaccination methods has been serum hemagglutination inhibition titers.

The aims of the present work were to determine the effect that the route of primary vaccination has on the appearance of antibody of each immunoglobulin class in secretions both before and after reexposure by the respiratory route, the duration of the local response, and the correlation of the development of local antibody levels with serum antibody. A sensitive solid-phase radioimmunoassay was developed in this laboratory to permit quantitation of low levels of antibody in secretions.

MATERIALS AND METHODS

Animals. Fifteen White Leghorn chickens were hatched from eggs purchased from Specific Pathogen Free Avian Supply, Norwich, Conn. The birds were raised in batteries maintained in rooms supplied with filtered air and under negative pressure.

Treatment. At 6 weeks of age, the birds were divided into three separate groups of five each: (i) five were inoculated intramuscularly (i.m.) with 0.2 ml of a stock B-1-type Lasota strain of ND containing $10^{8.45}$ 50% effective lethal doses per ml; (ii) five were inoculated intratracheally (i.t.) and intranasally with 0.4 and 0.1 ml, respectively, of the same stock of virus diluted 1:10 in sterile phosphate-buffered saline (PBS); and (iii) five remained unexposed to NDV and served as controls.

On days 4, 7, 10, 14, 21, and 28 after vaccination, saliva and blood were obtained from each bird. Thirty days after primary exposure to NDV, both groups were reinoculated i.t. with the same strain of NDV as that described above. A 0.6-ml amount of the vaccine stock diluted 1:3 in PBS was injected into the trachea

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of each bird. Serum and salivary samples were obtained from each bird on the same schedule as that after primary exposure.

Collection and testing of samples. Blood was obtained from the wing vein and serum was extracted from clotted blood. Each chicken was induced to salivate by injecting 0.5 to 0.7 ml of a 1% solution of pilocarpine nitrate (Sigma Chemical Co., St Louis, Mo.) in PBS via the wing vein. Saliva was aspirated from the oral cavity with a 10-ml syringe with a short piece of plastic tubing extending from an 18-gauge needle. Between 0.5 and 1.0 ml of the first saliva aspirated was placed in a tube on ice, diluted fourfold with PBS, and subjected to sonic treatment for 0.5 min at setting no. 3 (Sonifier model W185; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) to break up the mucin. The sample was centrifuged at 500 × *g* for 15 min to remove solid debris, and the supernatant was transferred to vials and frozen until assayed.

Serum anti-NDV antibody was quantitated by the hemagglutination inhibition (HI) assay (25), using 4 U of antigen. Salivary anti-NDV antibody was assayed by the plaque neutralization test (8). The levels of given classes of anti-NDV antibody in saliva, undiluted and diluted 1:2, were determined by the radioimmunoassay described below. Salivary samples were tested for the presence of immunoglobulins by the Ouchterlony method (29), using 1% agarose gel containing 2% polyethylene glycol (6,000 molecular weight) and class-specific antisera.

Radioimmunoassay. The solid-phase radioimmunoassay described previously (7, 14–16, 32) was modified for quantitation of class-specific anti-NDV immunoglobulins in chickens (technique to be reported elsewhere). Briefly, stocks of NDV antigen were prepared in cell culture. Monolayers of Vero monkey kidney cells were inoculated with a strain of NDV adapted to growth in Vero cells (kindly supplied by P. Lukert, Department of Medical Microbiology, University of Georgia, Athens). After 5 days of incubation, when about 50% of the monolayer showed cytopathic effects, the medium and cells were frozen and thawed three times and subjected to sonic treatment for 1 min on setting no. 5, and the virus was inactivated with β-propiolactone. The preparation was clarified, and the supernatant was harvested and frozen. Stocks of nonviral antigens were prepared to serve as negative controls. Allantoic fluid containing some whites was aspirated from eggs laid by dams free of NDV antibody, clarified by centrifugation, and frozen. Noninfected Vero monkey kidney cells were grown and processed as described above except that the cell pellet was washed two more times, resuspended in PBS, and frozen. Protein concentrations were determined by the method of Lowry et al. (22).

Flat-bottom polyvinyl microtiter plates were treated with a water-soluble solution of silicone (Siliclad; Clay-Adams, Inc., Parsippany, N.J.). An experimentally determined optimum dilution of antigen was made in PBS, and 0.05 ml was placed in each well. The antigens were dried in the wells overnight at room temperature. The antigens were fixed by filling the wells with a cold solution of 10% Formalin in PBS and incubating the plates at 4°C for 15 min. The Formalin solution was removed, and the plates were washed three times with PBS containing 10% fetal calf serum and 0.5% Tween-20 (buffer 1) before use.

Samples of serum or saliva were diluted in buffer 1. A 0.025-ml portion of each dilution was placed in duplicate wells of viral and control antigens. The plates were incubated in a humidified chamber at 37°C for 1 h. The samples were removed, and the plates were washed three times with PBS containing 1% fetal calf serum and 0.05% Tween-20 (buffer 2). Then 0.025 ml of an optimal dilution of ^{125}I-labeled anti-immunoglobulin antibody (in buffer 2) was added to each well, incubated as before for 1 h, and then washed thoroughly with PBS and air dried. The wells were cut out, and each one was placed in a 7-ml scintillation vial. Four milliliters of scintillation cocktail, 0.05% 2a70 (Research Products International Corp., Elk Grove Village, Ill.) in scintillation grade Toluene, was added to each vial, and the radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrophotometer, model 3380.

Nonspecific adsorptions of both antibody and ^{125}I-labeled anti-immunoglobulin were assessed by placing duplicate amounts of each dilution in wells containing concentrations of nonviral protein antigen equal to those placed in the viral antigen wells.

Preparation of antisera. Affinity-purified, class-specific goat antibodies to chicken immunoglobulins (immunoglobulin G [IgG] and IgM from serum and IgA from bile) were prepared, tested for specificity, and conjugated with fluorochromes as described previously (9, 10). Monospecific antibody was iodinated with ^{125}I by the chloramine-T oxidation procedure (11).

Immunohistological methods. After collection of the last saliva samples, two birds from each vaccination group were sacrificed. Tissues from the upper respiratory tract, oral cavities, and orbits of normal adult chickens were processed for immunohistological examination by the paraffin embedding technique of Sainte-Marie (33). Four-micrometer sections of each specimen were mounted on glass slides, deparaffinized, and rehydrated in PBS before staining. Each section was covered with a drop of fluorescein- or rhodamine-conjugated antibody to γ, μ, or α chains and incubated in a humidified chamber for 1 h at 37°C. Unbound antibody was rinsed off in a staining dish with three changes of PBS, 15 min each, and the slides were air dried. Specimens were mounted with a 10% Elvanol mounting medium and examined with a series 10 Microstar microscope (American Optical Corp., Buffalo, N.Y.) equipped with an incident-light fluorescent illuminator. Appropriate exciter and barrier filters were used for observing fluorescein and rhodamine conjugates. Photographs were taken with Panatomic X film (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

Antibody in egg whites. During preliminary experiments, NDV antigens grown in embryonated eggs and allantoic fluid proved to be suitable as virus and control antigens, respectively, in the radioimmunoassay. However, when radiolabeled anti-IgA or -IgM antisera were tested in this system, high levels of background

activity were obtained in the control antigen wells. Examination of the allantoic fluid of 12 eggs for the presence of immunoglobulins by the double diffusion test in gel with class-specific antisera revealed the presence of both IgA and IgM, but no IgG. A further analysis was made of the egg whites obtained from dams free of serum anti-NDV antibody and dams possessing serum anti-NDV neutralizing antibody. The whites from 6 eggs of each source were individually extracted, and the protein of each was precipitated by addition of an equal volume of saturated ammonium sulfate. The precipitate was resuspended in 1/5 the original volume of egg white. After dialysis against PBS, each sample was tested for NDV plaque-neutralizing activity. All 6 of the egg white samples obtained from dams having anti-NDV antibody had titers of 1:8. No neutralizing activity was detected in undiluted concentrates of egg whites from antibody-free birds. Consequently, to avoid interference by either specific or nonspecific antibodies in the egg whites, radioimmunoassays were performed with virus propagated in Vero monkey cells and extracts of noninfected cells as viral and control antigens, respectively.

Development of antibody in adult chicken salivas. The levels of specific classes of anti-NDV antibody and plaque-neutralizing antibody found in saliva and HI antibody found in serum of birds vaccinated either i.t. and intranasally or i.m. with NDV are given in Table 1 and Fig. 1 and 2. HI and plaque-neutralizing titers were each plotted as the geometric mean

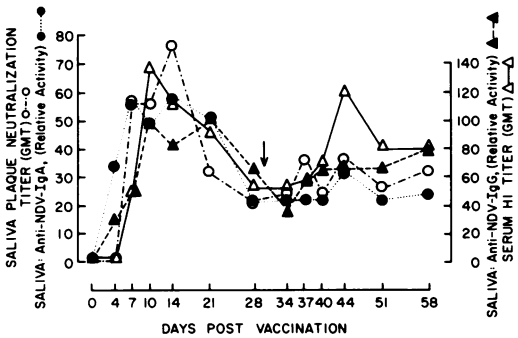

FIG. 1. *Antibody levels in serum (HI titer) and saliva (plaque neutralization, IgG, and IgA) of chickens vaccinated locally with NDV followed by local revaccination (arrow). GMT, Geometric mean titer.*

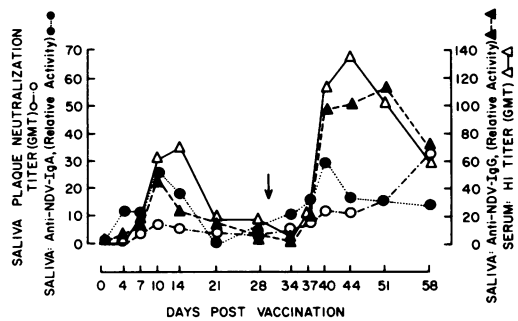

FIG. 2. *Antibody response in serum (HI titer) and saliva (plaque neutralization, IgG, and IgA) of chickens vaccinated i.m. with NDV followed by local revaccination (arrow). GMT, Geometric mean titer.*

TABLE 1. *Anti-NDV antibody levels in serum and saliva of chickens vaccinated i.m. or locally, via the respiratory tract, followed by local revaccination at 30 days after initial exposure*

Days after initial exposure	Serum HI titer[a]		Saliva					
			Plaque neutralization titer[a]		Anti-NDV IgA (relative activity)[b]		Anti-NDV IgG (relative activity)[b]	
	i.m.	Local	i.m.	Local	i.m.	Local	i.m.	Local
0	0	0	0	0	0	0	0	0
4	0	0	0	0	12 ± 3.0	33 ± 4.7	7 ± 2.3	29 ± 3.9
7	20 ± 1.8	46 ± 1.4	4 ± 2.3	55 ± 1.8	11 ± 2.7	57 ± 8.8	11 ± 4.2	50 ± 6.7
10	61 ± 1.9	139 ± 1.4	8 ± 1.8	56 ± 1.8	25 ± 4.5	47 ± 10.4	50 ± 1.2	100 ± 7.1
14	70 ± 1.4	113 ± 1.5	6 ± 1.6	76 ± 1.4	18 ± 1.7	58 ± 6.4	24 ± 2.6	83 ± 10.6
21	19 ± 4.5	92 ± 1.4	4 ± 1.8	32 ± 1.0	0.2 ± 0.3	49 ± 5.1	18 ± 2.8	100 ± 10.3
28	17 ± 1.4	53 ± 1.5	4 ± 2.2	22 ± 1.5	8 ± 1.0	21 ± 3.7	6 ± 1.5	66 ± 9.2
34	8 ± 2.0	53 ± 1.6	4 ± 1.2	25 ± 1.4	11 ± 1.4	22 ± 4.0	2 ± 0.3	36 ± 6.8
37	24 ± 3.3	57 ± 1.5	8 ± 3.8	37 ± 2.1	15 ± 2.5	23 ± 3.0	21 ± 2.0	56 ± 5.8
40	113 ± 3.8	70 ± 2.1	13 ± 1.5	24 ± 1.9	29 ± 3.6	22 ± 4.4	97 ± 11.7	64 ± 11.4
44	135 ± 1.9	122 ± 3.2	11 ± 2.0	37 ± 2.5	16 ± 2.9	32 ± 7.2	100 ± 20.5	66 ± 8.5
51	101 ± 2.2	80 ± 2.7	16 ± 2.1	27 ± 1.9	16 ± 3.8	22 ± 7.3	112 ± 23.1	67 ± 7.6
58	57 ± 4.4	80 ± 3.5	31 ± 2.1	32 ± 1.7	13 ± 2.5	25 ± 7.5	70 ± 5.4	79 ± 8.9

[a] Geometric mean titer ± standard error.

[b] Average ± standard error of the relative activity (average counts per minute) of the sample divided by the activity of a saliva standard multiplied by 100).

titer of four or five samples collected on a given day. The levels of anti-NDV IgG and anti-NDV IgA were each represented as the arithmetic mean of the counting ratios (the percentage of the activity of a test sample divided by the activity of a saliva standard) for samples collected on a given day after vaccination. No IgM was detected in fresh, undiluted saliva samples by the Ouchterlony method. Also, in a preliminary study with ^{125}I-labeled anti-IgM antibody, antibody against NDV could not be detected in fresh salivas obtained from birds 7 and 14 days after i.t. vaccination. Both of the above tests revealed the presence of IgG and IgA antibodies in saliva. Therefore, further assays for anti-NDV IgM were not performed in this study.

Among the birds vaccinated i.t. and intranasally, the levels of antibody activity followed each other very closely. After reaching maximum values between 10 and 14 days postvaccination, the levels dropped on day 28. After i.t. revaccination, there was a slight rise in the serum HI titer, which reached a maximum around 2 weeks after reexposure and then returned to a level slightly higher than that seen before revaccination. Antibody levels in saliva remained essentially unchanged after revaccination.

Antibody levels of the i.m.-vaccinated birds reached maximums about 10 to 14 days after exposure and then dropped to barely detectable levels by day 21. When compared with the i.t.-vaccinated group, the levels of serum HI antibody, salivary anti-NDV plaque-neutralizing antibody, and IgG antibody were about half as high as their maximum levels. Salivary anti-NDV IgA was barely detectable. Live virus was detected in salivary samples up to 7 days after i.t. revaccination of the i.m.-vaccinated birds. The vaccine virus was recognized by the appearance of plaques 2 days after the plaques of the velogenic strains of NDV had appeared in the neutralization test. No live vaccine virus was detected in salivas of i.t.-revaccinated birds which had been previously vaccinated i.t.

After i.t. revaccination of the i.m.-vaccinated group, a marked rise in serum HI antibody and salivary anti-NDV IgG occurred, reaching a peak about 14 days after reexposure. Only a slight rise in salivary plaque-neutralizing activity and a gradual rise in specific salivary IgA occurred after revaccination.

Immunohistology. Tissues from chickens of each vaccination group were examined for plasma cells with fluorochrome-labeled antibodies to μ, γ, and α chains to determine the source of the antibody found in salivary secretions. A sparse distribution of plasma cells was found in the lamina propria of the roof and floor of the oral cavity and the tongue. These cells were primarily of the IgG and IgM classes, with few IgA-positive plasma cells being detected. Salivary glands in the above tissues, however, contained exclusively IgA-positive plasma cells within the interacinous spaces. The largest numbers of plasma cells found in the head regions of the chickens were in the Harderian glands. All three classes of immunoglobulin-producing plasma cells were found in the interacinous spaces of the glands (Fig. 3). The majority of these cells contained IgG, whereas IgM- and IgA-containing plasma cells represented only a small proportion of the cells present.

DISCUSSION

Observations reported in this study indicate that IgG and IgA are the predominant immunoglobulins in saliva. These findings are in agreement with several other reports (19–21, 28). Immunohistological examination of various tissues of upper respiratory tracts revealed that IgG-containing plasma cells comprise the major portion of the plasma cells present in reimmunized birds. Both IgA- and IgM-containing cells, which were about equally prevalent, represented only a small proportion of the total number of plasma cells. Of all the tissues examined, the Harderian gland contained the highest density of plasma cells of all three classes. In unimmunized birds, the proportions of cells containing each of the three classes of immunoglobulin are about equal in the Harderian gland (unpublished data). The apparent higher proportion of IgG-containing plasma cells observed in this study may have been a result of local immunization. Bienenstock et al. (4) likewise found the Harderian gland to contain a small proportion of IgA-synthesizing plasma cells; but they reported that IgM cells were more prevalent than IgG-containing cells. This dense concentration of plasma cells in the Harderian glands and the ducts of the lateral nasal gland (1) as well as those distributed throughout the interacinous spaces of salivary glands and the lamina propria of the oral cavity most likely contribute to the secretions which discharge into the eyes, mouth, and nasal vestibule, thus bathing the upper respiratory tract and possibly the anterior end of the trachea with antibody. Mueller et al. (24) reported that sheep erythrocytes which were dropped into the eye of a bird could almost immediately be detected in the mouth. Therefore, the secretions and cells discharged from the Harderian glands, which empty into the orbit, can quickly find passage to the nasal and oral cavities via the lacrymal ducts. This same group demonstrated that antibody-producing cells appeared in the Harderian glands when antigen was introduced in the eye but not when it was

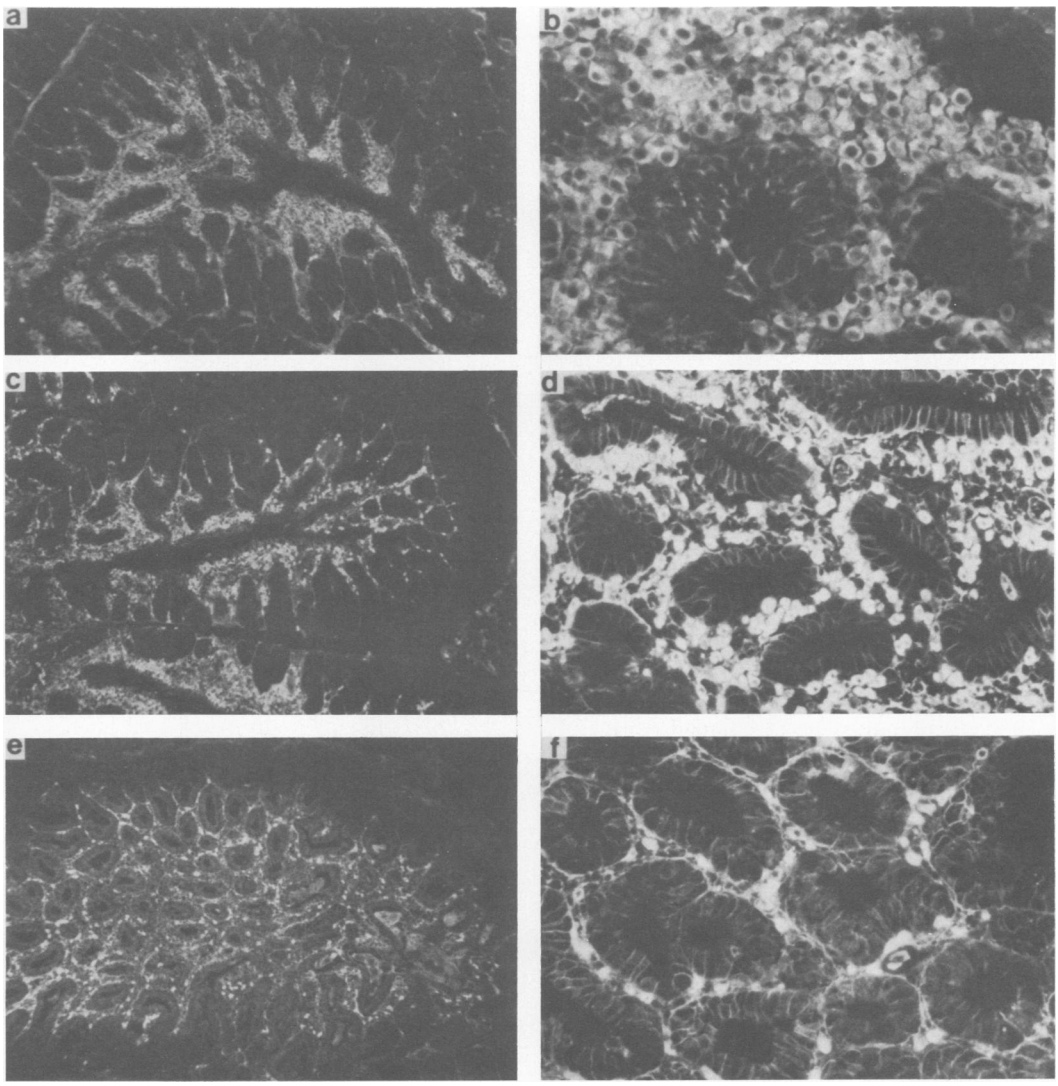

FIG. 3. *Sections of Harderian glands from NDV-revaccinated chickens, stained with fluorochrome-labeled antibody to (a, b) γ chains, (c, d) μ chains, and (e, f) α chains. Magnifications: (a) ×110, (b) ×400, (c) ×80, (d) ×370, (e) ×95, (f) ×370.*

given intravenously, thus indicating the local nature of this lymphoid system.

The effects of vaccination and revaccination with respect to the serum antibody response are comparable to those obtained in a similar study conducted by Beard and Easterday (2), in which i.m. and aerosol methods of vaccination and aerosol challenge were used. Birds vaccinated by the respiratory route had higher HI titers than did the i.m.-vaccinated birds. After aerosol challenge, they detected no appreciable rise in serum antibody levels in the aerosol-vaccinated birds but found a pronounced increase in the i.m.-vaccinated group. In the present study, the period between primary vaccination and reexposure was 7 days longer than that in their study; consequently, the serum antibody dropped to relatively lower levels, but the change in these levels after vaccination corresponds to their findings. They also observed that although no virus could be isolated from the tracheas of aerosol-vaccinated birds from 1 h to 8 days after challenge, virus was isolated from i.m.-vaccinated birds from 8 h to 4 days after challenge. Taken together, these observations provide a strong argument for the protection of the respiratory tract by local immune mechanisms which are activated by local application of antigen and

which neutralize virus upon reexposure, in the absence of detectable systemic antibody synthesis. The data presented herein suggest that this protection is in part mediated by specific antibody of the IgA and IgG classes found in the secretions of the respiratory tract.

i.t. vaccination produced a higher sustained level of antibody in saliva than did i.m. vaccination. Our failure to detect a significant rise in local antibody levels in i.t.-vaccinated birds after revaccination is most likely due to the neutralization of virus by antibody which was present at the time of challenge, but it may also be due to the failure of the local immune system to produce a secondary antibody response. An aspect of particular interest and worthy of further study is the duration of the local antibody synthesis and resistance to infection produced by local immunization, which in this and a previous report (2) persisted for at least 4 and 3 weeks, respectively.

After vaccination, the appearance of specific IgG and IgA antibody coincides with that of plaque-neutralizing activity in saliva. Although the initial rise in antibody levels may be the result of transuded serum antibody (10), the high levels of antibody which were sustained beyond the period of viral pathology in the i.t.-vaccinated group was most likely due to local production of antibody. The contribution of IgG and IgA to the salivary antibody corresponds to the relative occurrence of these plasma cells throughout the upper respiratory tracts of birds vaccinated i.t. and intranasally. These data do not define a role for the IgM-secreting cells, which we know are present in the Harderian gland in about equal proportions to the IgA- and IgG-secreting cells before antigen challenge. That it is minimal can be discerned from the lack of proliferation of the IgM cells after challenge and the absence of IgM in the saliva, as determined by a sensitive radioassay. Definition of their role requires further study.

The observations reported herein emphasize the importance of applying vaccine to the upper respiratory tract. This region appears to be particularly endowed with a dense population of B cells which are easily accessible to stimulation by local application of antigens. In the case of aerosol vaccination, droplet sizes should be used which can be easily retained in the upper respiratory tract. Small droplets may easily penetrate to the lower regions of the respiratory tract and thereby stimulate a humoral immune response (34) but fail to activate immunocytes in the upper respiratory tract. Consequently, aerosol generators which produce a wide range of droplet sizes may effect the best overall stimulation of both local and humoral immunities.

The detection of IgM and IgA but not IgG in the whites of unembryonated eggs is consistent with the findings of Rose et al. (31). However, the detection of anti-NDV activity in egg whites has not been previously reported. Bradly et al. (6) were not able to detect NDV-neutralizing activity in diluted (five times) egg whites obtained from eggs of immunized hens, although high levels of activity were found in the yolks. Also, Bornstein et al. (5) failed to detect HI antibody in whites of eggs from immunized hens. In the first report it is likely that any anti-NDV activity, if present, may have been diluted beyond detection, and in the second report the test used for detection was not sensitive enough. Similar negative results were obtained in this study when egg whites were assayed for HI activity. This further confirms the sensitivity of the radioimmunoassay described herein.

The results reported herein suggest that in birds, as in mammals, maternal antibody primarily of the IgA and IgM classes may be acquired by offspring by ingestion rather than by direct absorption into the fetal circulation. Maternal IgA and IgM present in the oviduct and incorporated in the egg whites mix with the amniotic fluid and bathe the external and internal surfaces of the embryo, providing transient protection for the emerging chick.

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LITERATURE CITED

1. **Bang, B. G., and F. B. Bang.** 1968. Localized lymphoid tissues and plasma cells in parocular and paranasal organ systems in chickens. Am. J. Pathol. **53**:735–751.
2. **Beard, C. W., and B. C. Easterday.** 1967. The influence of route of administration of Newcastle disease virus on host response. I. Serological and virus isolation studies. J. Infect. Dis. **117**:55–61.
3. **Beard, C. W., and B. C. Easterday.** 1967. The influence of the route of administration of Newcastle disease virus on host response. II. Studies on artificial passive immunity. J. Infect. Dis. **117**:62–65.
4. **Bienenstock, J., J. Gauldie, and D. Y. E. Perey.** 1973. Synthesis of IgG, IgA and IgM by chicken tissues: immunofluorescent and ^{14}C amino acid incorporation studies. J. Immunol. **111**:1112–1118.
5. **Bornstein, S., A. Rautenstein-Arazi, and Y. Samberg.** 1952. Some aspects of congenital passive immunity to Newcastle disease in chicks. I. The transfer of hemagglutinin inhibitors from the maternal yolk to the chick. Am. J. Vet. Res. **13**:373–378.
6. **Bradly, C. A., H. E. Moses, and E. L. Jungherr.** 1946. Transmission of antiviral activity via the egg and the role of congenital passive immunity to disease in chickens. Am. J. Vet. Res. **7**:333–342.
7. **Cleland, G. B., D. Y. E. Perey, and P. B. Dent.** 1975. Micro-radioimmunoassay for antibodies to Newcastle disease virus in the chicken. J. Immunol. **114**:422–425.
8. **Cunningham, C. H.** 1966. A laboratory guide to virology,

6th ed. Burgess Publishing Co., Minneapolis.
9. **Ewert, D. L., and C. S. Eidson.** 1977. Effect of bursectomy and depletion of immunoglobulin A on antibody production and resistance to respiratory challenge after local or systemic vaccination of chickens with Newcastle disease virus. Infect. Immun. **18**:146–150.
10. **Ewert, D. L., C. S. Eidson, and D. L. Dawe.** 1977. Factors influencing the appearance of antibody in tracheal washes and serum of young chickens after exposure to Newcastle disease virus. Infect. Immun. **18**:138–145.
11. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. The preparation of ^{131}I-labeled human growth hormone of high specific activity. Biochem. J. **89**:114–123.
12. **Heremans, J. F.** 1975. The secretory immune system. A critical appraisal, p. 376–385. *In* F. Milgrom and E. Neter (ed.), The immune system and infectious diseases: Proceedings. International Convocation Immunology, 4th, Buffalo, N.Y., June 1974. Karger, Basel.
13. **Holmes, H. C.** 1973. Neutralizing antibody in nasal secretions of chickens following administration of avian infectious bronchitis virus. Arch. Gesamte Virusforsch. **43**:235–241.
14. **Hutchinson, H. D., and D. W. Ziegler.** 1972. Simplified radioimunoassay for diagnostic serology. Appl. Microbiol. **24**:742–749.
15. **Hutchinson, H. D., and D. W. Ziegler.** 1974. Criteria for preparing, evaluating, and standardizing iodinated globulins for radioimmunoassay procedures. Appl. Microbiol. **28**:935–942.
16. **Hutchinson, H. D., D. W. Ziegler, and P. W. Feorino.** 1975. Radioimmunoassay for detection of antibodies to Epstein-Barr virus in human infectious mononucleosis serum specimens. J. Clin. Microbiol. **1**:429–433.
17. **Jungherr, E. L.** 1964. Pathogenicity of Newcastle disease virus for the chicken, p. 257–272. *In* R. P. Hanson (ed.), Newcastle disease virus: an evolving pathogen. University of Wisconsin Press, Madison.
18. **Kohn, A.** 1955. Quantitative aspects of Newcastle disease virus in infection: effect of route of infections on the susceptibility of chicks. Am. J. Vet. Res. **16**:450–457.
19. **Lebacq-Verheyden, A. M., J. P. Vaerman, and J. F. Heremans.** 1974. Quantification and distribution of chicken immunoglobulins IgA, IgM and IgG in serum and secretions. Immunology **27**:683–692.
20. **Leslie, G. A., and L. N. Martin.** 1973. Studies on the secretory immunologic system of fowl. III. Serum and secretory IgA of the chicken. J. Immunol. **110**:1-9.
21. **Leslie, G. A., and L. N. Martin.** 1974. The secretory immunologic system of fowl. IV. Serum and salivary immunoglobulins in normal, agammaglobulinemic and dysgammaglobulinemic chickens. Int. Arch. Allergy Appl. Immunol. **46**:834–841.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
23. **Malkinson, M., and P. A. Small, Jr.** 1977. Local immunity against Newcastle disease virus in the newly hatched chicken's respiratory tract. Infect. Immun. **16**: 587–592.
24. **Mueller, A. P., K. Sato, and B. Glick.** 1971. The chicken lacrimal gland, gland of Harder, caecal tonsil, and accessory spleens as sources of antibody-producing cells. Cell. Immunol. **2**:140–152.
25. **National Research Council.** 1971. Methods for examining poultry biologics and for identifying and quantifying avian pathogens. National Academy of Sciences, Washington, D.C.
26. **Ogra, P. L., and D. T. Karzon.** 1970. Role of immunoglobulins in the mechanism of mucosal immunity to virus infection. Pediatr. Clin. North Am. **17**:385–400.
27. **Ogra, P. L., R. B. Wallace, G. Vmana, S. S. Ogra, D. Kerr-Grant, and A. Morgg.** 1974. Implication of secretory immune system, p. 271–282. *In* J. Mestecky and A. R. Lawton III (ed.), The immunoglobulin A system. Plenum Publishing Corp., New York.
28. **Orlans, E., and M. E. Rose.** 1972. An IgA-like immunoglobulin in the fowl. Immunochemistry **9**:833–838.
29. **Ouchterlony, O.** 1949. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. **26**:507–515.
30. **Parry, S. H., and I. D. Aitken.** 1973. Immunoglobulin A in the respiratory tract of the chicken following exposure to Newcastle disease virus. Vet. Rec. **63**:258–259.
31. **Rose, M. E., E. Orlans, and N. Buttress.** 1974. Immunoglobulin classes in the hen's eggs: their segregation in yolk and white. Eur. J. Immunol. **4**:521–523.
32. **Rosenthal, J. D., K. Hayashi, and A. L. Notkins.** 1973. Comparison of direct and indirect solid-phase microradioimmunoassays for the detection of viral antigens and antiviral antibody. Appl. Microbiol. **25**:567–573.
33. **Sainte-Marie, G.** 1962. A paraffin embedding technique for studies employing immunofluorescence. J. Histochem. Cytochem. **10**:250–256.
34. **Waldman, R. H., and R. Ganguly.** 1974. The role of the secretory immune system in protection against agents which infect the respiratory tract, p. 283–294. *In* J. Mestecky and A. R. Lawton III (ed.), The immunoglobulin A system. Plenum Publishing Corp., New York.
35. **Zakay-Rones, Z., and R. Levy.** 1973. Immunologic response of chicks to inactivated Newcastle disease virus. Avian Dis. **17**:450–452.
36. **Zakay-Rones, Z., R. Levy, and G. Spira.** 1971 Local immunologic response to immunization with inactivated Newcastle disease virus. J. Immunol. **107**:1180–1183.