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Protective Immunity Against Newcastle Disease: The Role of Cell-Mediated Immunity

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SUMMARY. The role of cell-mediated immunity (CMI) in protection of birds from Newcastle disease was investigated by two different strategies in which only Newcastle disease virus (NDV)-specific CMI was conveyed without neutralizing antibodies. In the first strategy, selected 3-wk-old specific-pathogen-free (SPF) birds were vaccinated with either live NDV (LNDV), ultraviolet-inactivated NDV (UVNDV), sodium dodecyl sulfate–treated NDV (SDSNDV), or phosphate-buffered saline (PBS) (negative control) by the subcutaneous route. Birds were booster vaccinated 2 wk later and challenged with the velogenic Texas GB strain of NDV 1 wk after booster. All vaccinated birds had specific CMI responses to NDV as measured by a blastogenesis microassay. NDV neutralizing (VN) and hemagglutination inhibition (HI) antibody responses were detected in birds vaccinated with LNDV and UVNDV. However, birds vaccinated with SDSNDV developed antibodies that were detected by western blot analysis but not by the VN or HI test. Protection from challenge was observed only in those birds that had VN or HI antibody response. That is, birds with demonstrable CMI and VN or HI antibody response were protected, whereas birds with demonstrable CMI but no VN or HI antibody response were not protected. In the second strategy, birds from SPF embryos were treated *in ovo* with cyclophosphamide (CY) to deplete immune cells. The birds were monitored and, at 2 wk of age, were selected for the presence of T-cell activity and the absence of B-cell activity. Birds that had a significant T-cell response, but not a B-cell response, were vaccinated with either LNDV, UVNDV, or PBS at 3 wk of age along with the corresponding CY-untreated control birds. The birds were booster vaccinated at 5 wk of age and were challenged with Texas GB strain of NDV at 6 wk of age. All birds vaccinated with LNDV or UVNDV had a specific CMI response to NDV, VN or HI NDV antibodies were detected in all CY-nontreated vaccinated birds and some of the CY-treated vaccinated birds that were found to have regenerated their B-cell function at 1 wk postbooster. The challenge results clearly revealed that CY-treated birds that had NDV-specific CMI and VN or HI antibody responses to LNDV or UVNDV were protected, as were the CY-nontreated vaccinated birds. However, birds that had NDV-specific CMI response but did not have VN or HI antibodies were not protected from challenge. The results from both strategies indicate that specific CMI to NDV by itself is not protective against virulent NDV challenge. The presence of VN or HI antibodies is necessary in providing protection from Newcastle disease.

RESUMEN. Protección inmunitaria contra la enfermedad de Newcastle: El papel de la inmunidad mediada por células.

Se investigó el papel de la inmunidad mediada por células en la protección de las aves contra la enfermedad de Newcastle por medio de dos estrategias diferentes en las cuales se transmitió la inmunidad mediada por células específica contra Newcastle sin una respuesta humoral. En la primera estrategia, aves seleccionadas libres de patógenos específicos de tres semanas de edad fueron vacunadas vía subcutánea con un virus vivo de la enfermedad de Newcastle, un virus de Newcastle inactivado por medio de rayos ultravioleta, un virus de Newcastle tratado con sulfato dodecílico de sodio ó una solución salina buferada (PBS) como control negativo. Las aves fueron revacunadas dos semanas más tarde y desafiadas una semana después de la revacunación con la cepa velogénica Texas GB del virus de la enfermedad de Newcastle. Todas las aves vacunadas tuvieron respuesta inmunitaria mediada por células contra la enfermedad de Newcastle como se observó por medio de la prueba

de la blastogenesis. Los anticuerpos neutralizantes contra el virus de Newcastle y la respuesta de anticuerpos medidos por medio de la prueba de la inhibición de la hemoaglutinación fueron detectados en aves vacunadas con virus vivo de la enfermedad de Newcastle y con el virus inactivado por medio de rayos ultravioleta. Sin embargo, las aves que recibieron el virus tratado con sulfato dodecílico de sodio desarrollaron anticuerpos que fueron detectados por medio del análisis puntual Western pero no por medio de la virus neutralización o la inhibición de la hemoaglutinación. Se observó protección únicamente en las aves con respuestas de anticuerpos a las pruebas de virus neutralización o a la inhibición de la hemoaglutinación. Es decir, aves con inmunidad mediada por células demostrable y respuesta de anticuerpos detectada por virus neutralización o inhibición de la hemoaglutinación estuvieron protegidas, mientras que aves con inmunidad mediada por células demostrable pero que no mostraron respuesta de anticuerpos mediante la virus neutralización o la inhibición de la hemoaglutinación no estuvieron protegidas. En la segunda estrategia, aves nacidas de embriones libres de patógenos especificos fueron inoculadas *in ovo* con cìclofosfamida para deprimir las células B. Estas aves fueron examinadas y seleccionadas a las dos semanas de edad por la presencia de actividad de las células T y la ausencia de actividad de las células B. Las aves que tuvieron una respuesta de células T significante pero respuesta de células B no significante fueron vacunadas con el virus vivo, con el virus inactivado por medio de rayos ultravioleta ó con solución salina fisiológica a las tres semanas junto con el grupo control correspondiente de aves no tratadas con ciclofosfamida. Las aves fueron revacunadas a las 5 semanas de edad y fueron desafiadas con la cepa Texas GB de Newcastle a las 6 semanas de edad. Todas las aves vacunadas con el virus vivo o con el virus inactivado por medio de rayos ultravioleta tuvieron una respuesta immune mediada por células específica para el virus de Newcastle. La respuesta de anticuerpos medida por virus neutralización o inhibición de la hemoaglutinación fue detectada en todas las aves vacunadas y no tratadas con ciclofosfamida y en algunas aves vacunadas tratadas con ciclofosfamida que mostraron regeneración de la función de células B a la semana después de la revacunación. Los resultados del desafio revelaron claramente que las aves tratadas con ciclofosfamida que tuvieron respuesta immune mediada por células específica para Newcastle y respuesta de anticuerpos medida por virus neutralización o inhibición de la hemaglutinación para los virus vivos de la enfermedad de Newcastle, un virus de Newcastle inactivado por medio de rayos ultravioleta fueron protegidas como lo fueron las aves control vacunadas no tratadas con ciclofosfamida. Sin embargo, las aves que tuvieron respuesta inmunológica mediada por células especifica para Newcastle pero no tuvieron anticuerpos medidos por virus neutralización ó inhibición de la hemoaglutinación no fueron protegidas contra el desafio. Los resultados de estas dos estrategias indican que la respuesta inmunológica mediada por células por si sola no protege contra el desafío virulento del virus de Newcastle. La presencia de anticuerpos neutralizantes o inhibidores de la hemoaglutinación es necesaria en la protección contra la enfermedad de Newcastle.

Key words: cell-mediated immune response, cyclophosphamide, Newcastle disease virus

Abbreviations: CMF = calcium and magnesium free; CMI = cell-mediated immunity; Con A = concanavalin A; CY = cyclophosphamide; EID_{50} = 50% embryo infectious dose; HA = hemagglutination; HI = hemagglutination inhibition; LNDV = live Newcastle disease virus; LPS = lipopolysaccharide; MTT = 3-(4,5-dimethylthiazole-2-yl),2-5-diphenyltetrazolium bromide; ND = Newcastle disease; NDV = Newcastle disease virus; PBS = phosphate-buffered saline; SDS = sodium dodecyl sulfate; SI = stimulation index; SPF = specific-pathogen free; TBS = Tris-buffered saline; UVNDV = ultraviolet-inactivated Newcastle disease virus; VN = virus neutralization

Newcastle disease (ND) is an economically important viral disease of poultry and is of major concern worldwide (2). It occurs in a variety of avian species and may cause respiratory distress, diarrhea, cessation of egg production, nervous signs, depression, and high morbidity and mortality if not controlled (2). The severity of the disease produced by a virulent strain of Newcastle disease virus (NDV) has been found to be greatly influenced by the immune status of the host. The level of immunity against NDV determines the severity of the disease (4).

Both cellular and humoral immune responses have been suggested to play important roles

in the host's defense against NDV infection (5,10,11,14,16,17). Antibodies directed against surface glycoproteins of NDV have been reported to inactivate or neutralize the free virus. These antibodies inhibit virus attachment to the cell host receptors and prevent the spread of the virus from cell to cell (16). The antibody response to NDV occurs rapidly with detectable neutralizing antibodies, usually detected in the serum of birds within 4–6 days after vaccination with live attenuated vaccines (2).

Cell-mediated immunity (CMI) has been suggested to be an important factor in the development of protection in chickens vaccinated against ND (5,10,14,16,17). CMI has been reported as the first immunologic response, being detected as early as 2–3 days after ND vaccination (10). Early protection after vaccination can be demonstrated in the presence of low levels of antibodies or in the absence of detectable antibodies (11). Furthermore, recent studies with other paramyxoviruses have demonstrated that protective immunity is mediated by CMI, whereas virus-neutralizing antibodies have been found to play a minor role in protection (23). Although a protective role for cellular immunity has been suggested, a definitive relationship between protection and the cellular immune response has not been determined in the absence of a detectable antibody response (14,17).

The objective of this study was to ascertain whether CMI is a key component in the protection of chickens against ND. Two strategies were used to achieve this objective. The first strategy was to alter neutralizing epitopes on the virus by treating NDV with the denaturing agent sodium dodecyl sulfate (SDS). By this method, NDV-specific neutralizing or hemagglutination inhibition (HI) antibodies were not induced, but NDV-specific CMI was induced in immunocompetent birds. The second strategy was to deplete the humoral immune response of the bird while retaining the T-cell response and use intact NDV as an immunogen. This was achieved by injecting chicken embryos *in ovo* with cyclosphosphamide (CY).

MATERIALS AND METHODS

Eggs. Specific-pathogen-free (SPF) white leghorn eggs were purchased (Hy-Vac Co., Gowrie, IA). The chickens were hatched and housed in a facility designed for maintaining SPF status.

Media and reagents. CY (Cytoxan; Mead Johnson and Co., Evansville, IN) was obtained in a dry form containing active ingredients. An aqueous solution was prepared by reconstituting 2 g in 100 ml (20 mg/ml) of calcium and magnesium-free (CMF) phosphate-buffered saline (PBS) and filtering it through a 0.22-μm syringe filter.

A solution of RPMI 1640 supplemented with 25 mM HEPES (*N*-[2-hydroxyethyl]peperazine-*N'*-[2-ethanesultonic acid]) and L glutamine (Sigma Chemical Co., St. Louis, MO), penicillin (200 U/ml), and streptomycin (200 μg/ml) was used for washing and resuspending the cells, diluting the mitogens and antigen, and culturing the cells. Concanavalin A (Con A) (Sigma) was used as the T-cell mitogen. An aqueous solution was prepared by dissolving 100 mg in 10 ml of CMF-PBS and filtering it through a 0.22-μm syringe filter. Con A was used at a working concentration of 50 μg/ml for whole blood and at a concentration of 6 μg/ml for purified lymphocytes. Lipopolysaccharide (LPS) from *Salmonella typhimurium* (Sigma) was used as the B-cell mitogen. It was prepared in CMF-PBS as 1 μg/μl. All stock solutions were dispensed into small aliquots and stored at −20 C until used. LPS was used at a working concentration of 1 μg/ml for the whole blood blastogenesis microassay. A solution of MTT (3-[4,5-dimethylthiazole-2-yl],2-5-diphenyltetrazolium bromide) (Sigma) was prepared by dissolving 10 mg of MTT in 1 ml of CMF-PBS and solubilizing it by sonication. The solution was then filtered through a 0.45-μm syringe filter and stored at 4 C in a dark bottle. The HCl-isopropanol (0.04 N HCl-isopropanol) solution was prepared by adding 40 ml of 1 N HCl to 1 liter of isopropanol. The HCl-isopropanol was stored at room temperature in a lightproof bottle.

Virus propagation and purification. NDV purification was based on the method of Alexander and Collins (3). The lentogenic type B1, strain B1, of NDV was propagated in 9-day-old embryonated SPF chicker eggs. The embryonated eggs were inoculated by the chorioallantonic route and incubated for 5 days at 37 C. The allantoic fluid was clarified by centrifugation at 3000 × *g* for 30 min. The virus was pelleted by centrifugation at 50,000 × *g* for 2 hr. The pellet was resuspended in 0.01 M Tris-NaCl, pH 7.2, and applied to a discontinuous sucrose gradient made from 14 ml 50% (w/v) and 21 ml 20% (w/v) in 10 mM Tris, 0.1 M NaCl, 1 mM ethylenediaminetetraacetic acid, pH 7.4. After centrifugation for 2 hr at 50,000 × *g*, a visible band was observed at the sucrose gradient interface. The virus band was collected and pelleted at 50,000 × *g* for 2 hr. Then the pellet was resuspended in PBS. The purified virus was assayed for total protein concentration by a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), and the purity was assessed by Coomassie blue-stained polyacrylamide gel electrophoresis. The pu-

rified virus was inactivated by exposure to ultraviolet light for 40 min and tested for virus replication in the embryonic eggs. A concentration of 0.3 μg/ml of ultraviolet light-inactivated NDV (UVNDV) was used for measuring CMI in the blastogenesis microassay and 40 μg/bird was used for vaccination. The purified NDV was treated with an equal volume of 4% SDS and heated at 100 C for 2 min. The SDS-treated NDV (SDS-NDV) was mixed 1:1 (v/v) with incomplete Freund's adjuvant and used for vaccination.

The velogenic Texas GB strain of NDV was propagated in embryonic eggs (as above) and used for challenge at 10^2 50% embryo lethal dose/bird.

Preparation of purified lymphocytes. Three milliliters of blood was collected from each chicken by venipuncture in a syringe containing 20 units/ml of herpin. The blood was diluted 1:1 with an RPMI 1640 medium. The blood was layered on Lymphoprep™ (Accurate Chemical and Scientific Corp., Westbury, NY) and centrifuged at 800 × *g* for 15 min at room temperature. The cellular band at the medium/Lymphoprep™ interface was collected and washed twice with RPMI 1640 medium. The viable lymphocytes were counted by trypan blue dye exclusion after the addition of a 10-μl solution of trypan blue in 90 μl of physiological saline (0.15 M NaCl). After the cell concentration was adjusted to 5×10^6 lymphocytes/ml in RPMI 1640, the purified lymphocytes were used for measuring CMI to NDV.

CY treatment. The procedure for CY injection was performed as previously described (20). Briefly, eggs were injected with CY during days 16, 17, and 18 of embryonic development. An aqueous solution of CY (0.1 ml, 20 mg/ml) was injected into the air cell membrane by insertion into the hole with a 25-gauge ⅝-inch (16-mm) needle. The same procedure was used to inject the control eggs with CMF-PBS at day 16 of embryonic development and on the two succeeding days.

Colorimetric blastogenesis assay. The procedure for the blastogenesis microassay was performed similarly to the procedures previously described (15). Briefly, the assay was carried out in a 96-well, flat-bottomed tissue culture plate (Corning Laboratory Sciences Co., Corning, NY). Two hundred microliters of RPMI containing 6 μg/ml Con A, 0.3 μg/ml UVNDV, or media without antigen (control well) was dispensed in each well, and 10 μl of RPMI containing 10^5 purified lymphocytes was added to each well. The plate was incubated at 37 C for 93 hr in a humid atmosphere of 5% CO_2. At 93 hr of incubation period, 20 μl of MTT (10 mg/ml) was added in each well and the plate was reincubated for 3 hr. At 96 hr of incubation, the plate was centrifuged at 1000 × *g* for 10 min a room temperature. The supernatant was removed carefully, and 175 μl of 1 N HCl-isopropanol was added into each well to dissolve the formazan crystals. Then the plate was shaken for 20 min on a plate shaker (mini-orbital shaker; Bellco Biotechnology, Vineland, NJ), and the blood lymphocytes were thoroughly resuspended by multiple pipettings with a micropipetter. The plate was centrifuged at 1000 × *g* for 10 min. One hundred microliters of supernatant was transferred to the corresponding wells of a new 96-well plate. The absorbance of each well was measured by a microtiter enzyme-linked immunosorbent assay reader (Model EL310; BIO-TEK Instruments, Inc. Winooski, VT) at a wavelength of 550 nm. The response was reported as a stimulation index (SI) as calculated by the following formula:

$$SI = [(\text{mean absorbance of stimulated culture}) - (\text{mean absorbance of unstimulated culture})]/(\text{mean absorbance of unstimulated culture}).$$

Western blot. Purified NDV was solubilized in a gel sample buffer containing 0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 0.001% bromophenol blue and heated at 100 C for 2 min. The proteins were separated on a 10% polyacrylamide gel according to the Laemmli procedure (13), and the gel was electrophoresed at a constant voltage of 150 V for 4 hr. Part of the gel was stained with Coomassie brilliant blue G 250 (Pharmacia Biotech, Piscataway, NJ), destained with methanol, and fixed. The remaining portion of the gel was prepared for transfer onto a 0.45-μm nitrocellulose membrane (Trans-Blot Transfer Medium; Bio-Rad) for a western blot analysis. The transfer was carried out for 4 hr in a transfer buffer consisting of 25 mM Tris, 192 mM glycine, pH 8.3, and 20% (v/v) methanol at a constant voltage of 60 V. After transfer, the membrane was stained with 0.05% Ponceau S dye (Fisher Biotech, Fisher Scientific, Fair Lawn, NJ) in a 1% acetic acid solution for band visualization. The nitrocellulose membrane containing the separated proteins was cut into 7-mm strips, and each strip was placed into an individual well in an immunoblotting plate. An avidin–biotin immunobinding assay was used for developing the blot as previously described (6). Briefly, a nitrocellulose membrane containing the separated proteins was blocked with 3% skim milk in Tris-buffered saline (TBS) (500 mM NaCl, 20 mM Tris, pH 7.5) for 1 hr Antiserum was diluted 1:400 in TBS containing 0.3% skim milk and added to each strip. After 1 hr of incubation, secondary biotinylated antibodies (biotinylated anti-chicken immunoglobulin G; Vector Laboratories, Burlingame, CA) at a dilution of 1:5000, streptavidin at a dilution of 1:2000, and the chromogen (4-chloro-1-naphthol; Sigma) were subsequently added. The nitrocellulose membrane was washed and blocked between steps. Then the im-

munoblotting plate was incubated in the dark until a color reaction developed against the white (nitrocellulose) background, and the reaction was stopped by washing with TBS.

Sample collection for virus isolation. Cotton-tipped applicators were used for swabbing the trachea. After tracheal swabbing, each cotton applicator was placed into 2 ml of tryptose phosphate broth containing penicillin and streptomycin and then frozen (−70 C). On the day of egg inoculation, the tubes were thawed and the swabs were removed. The remaining fluid was centrifuged at 1000 × *g* for 10 min and the supernatant was collected for embryo inoculation. Five 9-day-old embryonated eggs for each tracheal swab sample were inoculated via the chorioallantoic sac with 0.1 ml per embryo. Then the eggs were incubated at 37 C for 7 days. The presence of NDV was detected by hemagglutination (HA) with 1% turkey erythrocytes.

HI test. The HI test was performed as previously described (1,22). Briefly, twofold serial dilutions of serum were made in a 96-well, round-bottom microtiter plate containing 50 μl of PBS in the first row and 50 μl of NDV antigen (10 HA units) in the remaining 11 rows. Serum dilutions ranged from 1:2 to 1:2048. The antigen serum mixture was incubated for 30 min at 37 C. Then, 50 μl of a 0.05% turkey erythrocyte suspension was added to each well and reincubated for 30 min. A positive serum, a negative serum, erythrocytes, and antigens were also included as controls. The highest dilution of serum causing complete inhibition was considered the endpoint. The geometric mean titer was expressed as reciprocal $\log_2$ values of the highest dilution that displayed HI.

Virus neutralization (VN) test. The VN test was performed as previously described (19). Briefly, 50 μl of medium was added to 50 μl of the serum samples. Twofold serial dilutions were from 1:2 to 1:1024. One hundred 50% tissue culture infectious doses of the Texas GB strain of NDV were mixed with equal volumes of the serum dilutions. After a 1-hr incubation period, 100 μl of the virus–serum mixture were transferred into 96-well culture plates containing monolayers of the swine testicular cell line and incubated at 37 C for 72 hr. The plates were examined for cytopathic effect (CPE) to confirm the presence of the virus. Positive and negative serum controls were also included in the experiment. All samples were tested in quadruplicate. The 50% neutralizing endpoint was calculated by the method of Reed and Muench (18).

Experimental design. In the first experiment, four groups of 3-wk-old SPF chickens were injected subcutaneously with approximately 40 μg/bird of either LNDV, UVNDV, SDS-NDV, or PBS. The birds were booster vaccinated at 5 wk of age. Blood samples were collected weekly for an evaluation of cellular and humoral immune response. The birds were challenged 1 wk after booster vaccinations with 10^2 50% embryo infectious dose (EID_{50}) of Texas GB strain NDV that was administered intramuscularly. The birds were observed for 2 wk after challenge. Tracheal swabs were taken at 4 days postinoculation and used for virus isolation. Two trials were conducted, with 12 birds per group in the first trial and 10 birds per group in the second trial.

Birds hatched from SPF *in ovo* CY-treated and CY-nontreated eggs were used in the second experiment. At 2 wk of age, blood samples were taken from all *in ovo* CY-treated and CY-nontreated birds, and the MTT blastogenesis microassay was performed with B- and T-cell mitogens. Statistical analysis was performed with *t*-tests to select those birds that had a significant T-cell response and no significant B-cell response. At 3 wk of age, CY-treated birds that had a significant Con A (T cell) response and no significant LPS (B cell) response were vaccinated subcutaneously with approximately 40 μg/bird of either live NDV (LNDV) or UVNDV or injected with PBS. Similarly, the CY-untreated birds that were Con A (T cell) and LPS (B cell) responsive were vaccinated subcutaneously with 40 μg/bird of either LNDV, UVNDV, or PBS. The chicks were boostered at 5 wk of age, and blood samples were collected weekly for evaluation of cellular and humoral immune response. Challenge testing was administered intramuscularly 1 wk postbooster with 10^2 EID_{50} of the Texas GB strain of NDV per bird, and the chicks were observed for 2 wk afterward. Tracheal swabs were taken 4 days postinoculation and used for virus isolation.

Two trials were conducted. In the first trial, 10 CY-treated chickens were vaccinated with UVNDV and five were used for the other groups. Birds vaccinated with LNDV were not included in the first trial. In the second trial, 12 birds were used per group.

Statistical evaluation. Student *t*-tests were conducted to test the mitogenic response of each CY-treated bird and CY-nontreated bird to Con A and LPS mitogens. SI values were used for statistical evaluation of CMI response. The SAS statistical software (SAS Institute, Inc., Cary, NC) was used to compare the average SI of four birds from each group vaccinated with NDV vaccine and those from unvaccinated groups. Duncan's multiple range test was used to determine the differences in CMI among vaccinated and control groups, and statistical significance was expressed as highly significant ($P < 0.01$), significant ($P < 0.05$), or not significant ($P > 0.05$). Correlations between HI, CMI, and protection for the two experiments were determined by linear regression analysis and expressed as a correlation coefficient (r) for which $r > 0$ indicated a positive linear relationship and $r < 0$ indicated a negative relationship.

Table 1. Antibody titers to NDV as measured by the HI and VN assays of birds vaccinated with live, UV-inactivated, or SDS-treated NDV preparations.

		HI titer[A]			VN titer[A]
		Postvaccination		Postbooster	Postbooster
Trial	Treatment	10 days	14 days	10 days	10 days
1	LNDV	4.4 (3–6)[B]	6.1 (6–7)	9.2 (9–10)	9.62
	UVNDV	0	3 (0–4)	6.2 (5–8)	6.02
	SDS-NDV	0	0	0	<2
	PBS control	0	0	0	<2
2	LNDV	5 (5–6)	7.3 (6–9)	9.5 (9–10)	9.5
	UVNDV	0	2.8 (2–5)	5.2 (5–7)	5.5
	SDS-NDV	0	0	0	<2
	PBS control	0	0	0	<2

[A]Geometric mean titers of responding birds expressed as reciprocal $\log_2$.
[B]Numbers in parentheses indicate the range of HI titers within the group.

RESULTS

The results from trials 1 and 2 of Experiment 1 (Table 1) revealed that chickens vaccinated with LNDV and UVNDV produced detectable antibody titers as measured by the HI test and antibody titers increased subsequent to booster. However, chickens vaccinated with SDS-NDV did not produce detectable levels of specific antibody to NDV as measured by the HI assay. Similarly, the VN results (Table 1) revealed that virus-neutralizing antibodies were detected in all the chickens inoculated with LNDV and UVNDV but not in chickens vaccinated with SDS-NDV or uninoculated control birds. Western blot tests revealed that birds vaccinated with LNDV, UVNDV, and SDS-NDV had positive sera that reacted with NDV polypeptides. The western blot results of 10 individual birds vaccinated with SDS-NDV indicated that all the birds produced antibodies to the nucleoprotein/phosphoprotein NDV proteins (Fig. 1). However, the antibody response to the other NDV proteins varied between individual birds.

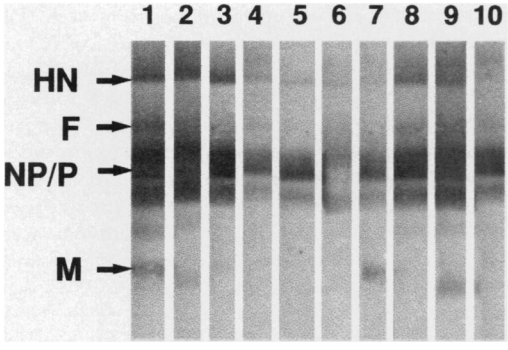

Fig. 1. Western blot results from 10 birds vaccinated with SDS-NDV. The NDV polypeptides are indicated as follows: HN = hemagglutinin neuraminidase, F = fusion, NP/P = nucleoprotein/nucleocapsid associated protein, M = matrix.

Table 2 shows the HI titers of sera from trials 1 and 2 of Experiment 2. The results revealed that *in ovo* CY-treated birds that were vaccinated with UVNDV did not produce detectable antibody titers during the first and second weeks postvaccination. However, antibody titers were detected in three birds at 1 wk postbooster and in all CY-nontreated birds that were vaccinated with LNDV and some of the birds vaccinated with UVNDV by 2 wk postvaccination. In addition, antibody titers increased in all NDV-vaccinated, CY-nontreated birds subsequent to booster vaccinations.

The VN results (Table 2) also revealed that all CY-treated vaccinated birds, with the exception of those birds that responded to the HI test, had no serum antibody response. However, a substantial response was detected in the CY-nontreated vaccinated birds.

The results from the blastogenesis microassays that were performed on the samples of four birds from each group are displayed in Tables 3 and 4. The results of the first experiment revealed that those birds vaccinated with LNDV, UVNDV, and SDS-NDV had a significant CMI response to NDV as compared with the control groups (Table 3). No significant differences were found between the three inoculation groups. Control birds did not produce a specific CMI blastogenesis response to NDV.

Table 2. Antibody response of CY-treated and CY-nontreated birds vaccinated with live or UV-inactivated NDV.

		HI titer			VN titer
		Postvaccination[B]		Postbooster[B]	Postbooster[C]
Trial	Treatment[A]	8 days	14 days	10 days	10 days
1	CY-UVNDV	1/10	0/10	3/10 (2.67)[D]	2.3
	CY-PBS	0/5	0/5	0/5	<2
	C-UVNDV	0/5	3/5 (3)	5/5 (5.4)	5.6
	C-PBS	0/5	0/5	0/5	<2
2	CY-LNDV	0/12	0/12	3/12 (3.6)	2.3
	CY-UVNDV	0/12	0/12	3/12 (2.6)	3.1
	CY-PBS	0/12	0/12	0/12	<2
	C-LNDV	12/12	12/12 (6.1)	12/12 (9.2)	9.62
	C-UVNDV	0/12	4/12 (3)	12/12 (6.2)	6.02
	C-PBS	0/12	0/12	0/12	<2

[A]CY-LNDV = CY-treated birds vaccinated with LNDV; CY-UVNDV = CY-treated birds vaccinated with UVNDV; CY-PBS = CY-treated birds injected with PBS; C-LNDV = control birds vaccinated with LNDV; C-UVNDV = control birds vaccinated with UVNDV; C-PBS = CY control birds injected with PBS.

[B]Number of birds having HI titers >1:2.

[C]Geometric mean titer of responding birds expressed as reciprocal $\log_2$.

[D]Numbers in parentheses indicate the geometric mean titer of responding birds expressed as reciprocal $\log_2$.

The results of the second experiment revealed that the birds vaccinated with UVNDV or LNDV had specific CMI responses to NDV that were significantly higher than that of the PBS-injected birds (Table 4).

The challenge results from the first and second experiments revealed that the birds that did not produce VN or HI antibody responses to NDV developed clinical signs of ND and died by 3 or 4 days postchallenge (Tables 5, 6). Because of mortality, tracheal swab samples could not be taken from birds that perished. The LNDV and UVNDV groups did not develop clinical signs of ND nor was NDV recovered from their tracheas.

Statistical analyses of the data indicated a high correlation between the presence of VN or

Table 3. Lymphocyte blastogenesis results from birds vaccinated with live, UV-inactivated, or SDS-treated NDV preparations.

Trial	Treatment	Mean SI[A] (±SD)
1	LNDV	$0.640^{a} \pm 0.233$
	UVNDV	$0.5746^{a} \pm 0.153$
	SDS-NDV	$0.436^{a} \pm 0.052$
	PBS control	$0.07^{b} \pm 0.195$
2	LNDV	$0.7151^{a} \pm 0.443$
	UVNDV	$0.250^{a} \pm 0.156$
	SDS-NDV	$0.4575^{a} \pm 0.059$
	PBS control	$0.043^{b} \pm 0.052$

[A]Average stimulation index of four birds. Values within the same column having different lowercase superscripts are significantly different ($P < 0.05$) as measured by Duncan's multiple range test.

Table 4. Lymphocyte blastogenesis test results from CY-treated and CY-nontreated birds vaccinated with live or UV-inactivated NDV preparations.

Trial	Treatment[A]	Mean SI[B] (±SD)
1	CY-UVNDV	$0.5217^{a} \pm 0.0519$
	CY-PBS	$0.0325^{b} \pm 0.0178$
	C-UVNDV	$0.7772^{a} \pm 0.442$
	C-PBS	$0.045^{b} \pm 0.032$
2	CY-LNDV	$0.610^{a} \pm 0.108$
	CY-UVNDV	$0.77805^{a} \pm 0.173$
	CY-PBS	$0.0645^{b} \pm 0.142$
	C-LNDV	$0.640^{a} \pm 0.233$
	C-UVNDV	$0.575^{a} \pm 0.1539$
	C-PBS	$0.070^{b} \pm 0.195$

[A]CY-LNDV = CY-treated birds vaccinated with LNDV; CY-UVNDV = CY-treated birds vaccinated with UVNDV; CY-PBS = CY-treated birds injected with PBS; C-LNDV = control birds vaccinated with LNDV; C-UVNDV = control birds vaccinated with UVNDV; C-PBS = control birds injected with PBS.

[B]Average stimulation index of four birds. Values in the same column having different lowercase superscripts are significantly different ($P < 0.05$) as measured by Duncan's multiple range test.

Table 5. Challenge test results of chickens vaccinated with live, UV-inactivated, or SDS-treated NDV after challenge with the Texas GB isolate of NDV.

Trial	Group	n^A	Mortality[B]	Virus shedding[C]
1	LNDV	12	0/12	Neg.
	UVNDV	12	0/12	Neg.
	SDS-NDV	12	12/12	ND
	PBS control	12	12/12	ND
2	LNDV	10	0/10	Neg.
	UVNDV	10	0/10	Neg.
	SDS-NDV	10	10/10	ND
	PBS control	10	10/10	ND

[A]Number of birds in each group.
[B]Number of dead birds/total challenged birds.
[C]Neg. = no virus shedding; ND = not done.

Table 6. Challenge test results of CY-treated and CY-nontreated birds vaccinated with LNDV or UVNDV. Birds were challenged with the Texas GB strains of NDV.

Trial	Group[A]	n^B	Mortality[C]	Virus shedding[D]
1	CY-UVNDV	10	7/10	0/3
	CY-PBS	5	5/5	ND
	C-UVNDV	5	0/5	Neg.
	C-PBS	5	5/5	ND
2	CY-LNDV	12	9/12	0/3
	CY-UVNDV	12	9/12	0/3
	CY-PBS	12	12/12	ND
	C-LNDV	12	0/12	Neg.
	C-UVNDV	12	0/12	Neg.
	C-PBS	12	12/12	ND

[A]CY-LNDV = CY-treated birds vaccinated with LNDV; CY-UVNDV = CY-treated birds vaccinated with UVNDV; CY-PBS = CY-treated birds injected with PBS; C-LNDV = control birds vaccinated with LNDV; C-UVNDV = control birds vaccinated with UVNDV; C-PBS = control birds injected with PBS.
[B]Number of birds in each group.
[C]Number of dead birds/total challenged birds.
[D]Neg. = no virus shedding; ND = not done.

HI antibody and protection ($r = 0.91$), whereas intermediate correlation was found between the presence of specific CMI to NDV and protection ($r = 0.47$).

DISCUSSION

Various approaches have been used for identifying the specific components of the immune system involved in protection (14,17,23). One approach has been to destroy one component of the immune system and then determine if protective immunity can be attained from the intact component (14,17). Another approach has been to passively transfer immunity from an immune donor to a nonimmune recipient (23). In this study, CY was used as an immunosuppressive agent to deplete B lymphocytes and repress humoral immunity (8,20). In addition, immune-competent birds were vaccinated with denatured NDV proteins in order to elicit a NDV-specific CMI response without inducing NDV HI or neutralizing antibodies. The results of this study indicated a high positive correlation between the presence of HI or VN NDV antibodies and protection from virulent NDV challenge. The presence of a NDV-specific CMI response in the absence of NDV HI or VN antibodies did not protect birds against virulent NDV challenge. CY-treated birds that were vaccinated with LNDV or UVNDV at 3 wk of age and developed a specific NDV CMI response, but did not produce NDV VN or HI antibodies, were susceptible to virus challenge. A small number of CY-treated birds that survived the virus challenge had developed an antibody response to NDV 1 wk postbooster. The antibody titers to NDV in the CY-treated birds that were protected were low as measured by the HI test. However, protection can be demonstrated in the presence of very low levels of antibody (5). Gough and Alexander (11) found 100% protection in chickens with low antibody titers (from less than 1:2 to 1:32) as measured by HI. Intermediate correlation was demonstrated between protection from challenge and the presence of NDV-specific CMI. However, the data analyzed were generated from fully competent birds as well as immune compromised birds. That is, those birds that were protected and had ample CMI responses also had antibodies to NDV.

The results of this study also demonstrated the importance of the protective epitopes that induce specific antibody response to NDV in protection. All sera from chickens immunized with SDS-NDV had antibodies that were detected by the western blot analysis (see Fig. 1) but not with the HI test or VN test. Those birds not having NDV-specific HI or VN antibodies were not protected from challenge. We

hypothesized that SDS treatment of the virus may have destroyed the conformational epitopes needed for inducing virus-neutralizing activity. This result has been reported to occur with other viruses. For example, immunizing chickens with denatured proteins of infectious bursal disease virus rendered the birds incapable of inducing virus-neutralizing antibodies (9).

A lymphocyte blastogenesis microassay was used in this study as an indicator of CMI. That CMI was induced when the chickens were vaccinated with ND vaccine was evident in the experiments. All birds, including the CY-treated and CY-nontreated birds that were vaccinated with UVNDV, LNDV, or SDS-NDV, elicited specific CMI as measured by the MTT blastogenesis microassay. No statistical differences were observed among any of the vaccinated groups. The results of the blastogenesis assays in the present study support the conclusions of previous studies (10,12). Ghumman and Bankowski (10) found that birds vaccinated with LNDV or inactivated ND vaccine elicited CMI as early as the second day after vaccination. Denatured protein was found to be capable of inducing mitogenic responses of peripheral blood lymphocytes from cattle immunized with bovine herpesvirus 1 as detected in the lymphocyte proliferation assay (12).

The quantity of NDV antigens used in the *in vitro* blastogenesis microassay was less than that reportedly used in other studies (10,14). Preliminary studies performed in our laboratory indicated that using a low concentration (0.3–0.6 μg/ml) of purified UVNDV produced a specific response without producing a nonspecific response, thus averting the need to eliminate any nonspecific reactivity when using NDV with the blastogenesis microassay as an indicator for NDV-specific CMI (21).

One interpretation from the results of this study is that antibodies are key components for protective immunity to ND. This interpretation might be considered contradictory to other reports that have emphasized the importance of CMI as a key component in protection (10,11,14,17). For example, Marino and Hanson (14) reported that *in ovo* bursectomized birds vaccinated with NDV were protected against virus challenge. Although their findings suggested a protective role for cellular immunity, they may have overlooked the importance of antibody-mediated protection. In their study, vaccinated bursectomized birds also developed antibodies but at much lower titers than control nonbursectomized vaccinated birds. The low titer in bursectomized birds was significantly lower and was assumed to be nonprotective. Therefore, protection was attributed to CMI.

Several research findings have supported the importance of CMI in controlling other paramyxovirus infections. Young *et al.* (23) found that the immune response to simian virus 5 was interposed entirely by CMI and serum-neutralizing antibodies that played only a minor role in protection. This may be explained in part by the fact that simian virus 5 establishes persistent nonlethal infections in infected cells, and *in vivo* spread of the virus occurs by cell-to-cell fusion. In such circumstances, the cytotoxic T cell is required to lyse the infected cells and eliminate the infection. Thus, the net effect of cytotoxic T cell activity is preventing further spread of the virus and terminating infection. This mechanism may not be applicable to NDV because NDV replicates rapidly, enabling large amounts of the infectious virus to be released quickly from infected cells before an effective immune response can be made. With such an infection, the cellular-immune response is unlikely to be sufficiently rapid to significantly alter the peak titer of the virus achieved in the host, whereas neutralizing antibodies, which are more successful in restricting the replication of viral infection and preventing the spread of virus within infected tissues, are more likely to be protective (2). Furthermore, recent work with the influenza virus has demonstrated that animals rendered deficient in cytotoxic or helper T cell responses were able to clear the influenza virus infection in a manner similar to their fully immunocompetent counterparts, suggesting that antibodies might participate in the clearance of viral infection (7). However, the role of CMI in limiting virus replication and/or shedding should not be discounted. The results of this study revealed that birds that were vaccinated with either the live or the inactivated preparation of intact NDV were protected from challenge and did not shed virus from their tracheas. Because the birds were administered the vaccines by the subcutaneous route, the fact that the birds did not shed virus from their tracheas cannot be attributed to direct stimulation of the mucosal or local immune response (as one might expect when administering NDV

vaccines by spray administration). Although in this study we made no attempt to explore the mechanism(s) involved in tracheal virus shedding, the data suggest that a CMI response may have been involved.

In conclusion, the results of this study support the concept that humoral immunity to NDV is a key component in the protection against ND. Therefore, vaccination programs should be directed toward eliciting and maintaining high antibody levels to NDV in flocks of birds.

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