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A Technique for Inducing B-Cell Ablation in Chickens by In Ovo Injection of Cyclophosphamide

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SUMMARY. The effect of cyclophosphamide (CY) treatment *in ovo* on avian B and T cells was studied. CY was injected *in ovo* on the 16th, 17th, and 18th days of incubation. Blood samples were collected periodically from CY-treated and nontreated birds after hatch and were used to measure blood lymphocyte responses to the T-cell and B-cell mitogens, concanavalin A and lipopolysaccharide (LPS), respectively. Additionally, flow cytometric analysis was used to determine the presence of B and T cells in peripheral blood, and birds were vaccinated with Newcastle disease virus (NDV) antigen at 3 wk of age and booster vaccinated at 5 wk of age. CY treatment reduced hatchability by 35%–40%, increased mortality by 3%–5% within the first 2 wk of life, and induced a significant retardation in body weight gains. At 2 wk of age, approximately 50% of CY-treated birds were devoid of B-cell mitogenic responsiveness while demonstrating significant T-cell mitogenic responsiveness. However, B-cell responses were observed at 4 and 6 wk from a small percentage of birds that were originally T-cell responsive and B-cell nonresponsive at 2 wk of age. Flow cytometric analysis of peripheral blood lymphocytes revealed that CY-treated birds had significantly less B cells (or were devoid of B cells) than the corresponding nontreated control birds. However, no significant difference in the T-cell percentage was observed between CY-treated and nontreated birds.

CY-treated birds did not produce detectable antibodies specific for NDV during the first and second weeks postvaccination, as demonstrated by hemagglutination inhibition assay. However, antibodies were detected in some CY-treated birds 10 days postbooster. Those antibody-positive birds were found to be the same birds that had subsequently responded to the LPS mitogen on the blastogenesis microassay. This study indicates the importance of monitoring the B- and T-cell responses in CY-treated birds to identify those birds in which B-cell regeneration may have occurred.

RESUMEN Técnica para la inducción de la destrucción de las células B en pollos mediante la inyección de ciclofosfamida in ovo.

Se estudió el efecto de la inyección de ciclofosfamida *in ovo* al dia 16, 17 y 18 de incubación sobre las células aviares B y T. Después del nacimiento se tomaron muestras de sangre periódicamente de aves tratadas y no tratadas con ciclofosfamida para medir las respuestas de los linfocitos sanguíneos a los mitógenos de las células T y B, concanavalina A y lipopolisacáridos, respectivamente. Además, se utilizó el análisis de flujo citométrico para determinar la presencia de células B y T en la sangre periférica y se vacunaron las aves con el virus de la enfermedad de Newcastle a las 3 semanas de edad y se revacunaron a las cinco semanas. El tratamiento con ciclofosfamida redujo el porcentaje de nacimientos en un 35%–40%, aumentó la mortalidad en un 3%–5% durante las 2 primeras semanas de vida y produjo un retardo significante en la ganancia de peso corporal. A las 2 semanas de edad, aproximadamente el 50% de las aves tratadas con ciclofosfamida no mostraron respuesta al mitógeno de las células B pero mostraron una respuesta fuerte a los mitógenos de las células T. Sin embargo, se observaron respuestas de las células B a las semanas 4 y 6 en un porcentaje pequeño de aves que a las 2 semanas de edad mostraron inicialmente respuesta de las células T pero no de las células B. El análisis de flujo citométrico de los linfocitos de la sangre periférica mostró que las aves tratadas con ciclofosfamida no tuvieron o tuvieron significantemente menor cantidad de células B que las aves controles correspondientes no tratadas. Sin

embargo, no se observaron diferencias significantes en el porcentaje de células T entre los grupos tratados y no tratados con ciclofosfamida.

Las aves tratadas con ciclofosfamida no produjeron anticuerpos detectables específicos contra el virus de la enfermedad de Newcastle durante la primera y segunda semana después de la vacunación, como se demostró mediante la prueba de la inhibición de la hemaglutinación. Sin embargo, se detectaron anticuerpos en algunas de las aves tratadas con ciclofosfamida 10 dias después de la revacunación. Estas aves positivas para anticuerpos contra el virus de Newcastle resultaron ser las mismas que respondieron al mitógeno lipopolisacárido en la microprueba de blastogenesis. Este estudio indica la importancia del seguimiento de las respuestas de las células B y T en aves tratadas con ciclofosfamida para identificar aquellas aves en las cuales pueda haber ocurrido regeneración de las células B.

Key words: cyclophosphamide, Newcastle disease virus, humoral immune response, immunosuppressive

Abbreviations: CMF-PBS = calcium- and magnesium-free phosphate-buffered saline; CMI = cell-mediated immunity; Con A = concanavalin A; CY = cyclophosphamide; FITC = fluorescein isothiocyanate; HI = hemagglutination inhibition; IgG = immunoglobulin G; IgM = immunoglobulin M; LPS = lipopolysaccharide; MAb = monoclonal antibody; MTT = 3-(4,5-dimethylthiazole-2-yl),2-5-diphenyltetrazolium bromide; NDV = Newcastle disease virus; PBL = peripheral blood lymphocytes; PBS = phosphate-buffered saline; SPF = specific-pathogen free; UV = ultraviolet; UV-NDV = ultraviolet-inactivated Newcastle disease virus

Cyclophosphamide (CY), a tumoricidal agent, is a nonspecific immunosuppressant agent affecting primarily antibody-mediated immunity (7,8). CY has been used experimentally as an immunosuppressant to elucidate some aspects of the immune response such as the role of cell-mediated immunity (CMI) in protection (6,9,12,13,15,16). In the chicken, CY given in newly hatched birds leads to severe and permanent deficiency in the humoral immune response. Eskola *et al.* (7) found that CY given *in ovo* caused severe humoral immunodeficiency when administered on days 14–16 or on days 16–18 of incubation. The suppressive effect, however, was found to be reversible, and the functional and morphologic recovery of antibody-producing tissue occurred after 10 wk post-treatment (7,14). CMI was also found to be initially affected, but regeneration of the T-cell response occurred more rapidly. T-cell responsiveness was reported to be suppressed for less than 2 wk (10,14).

The CY method for ablating B cells has been shown to be limited by the early recovery of antibody-producing organs, thus providing only temporary immunosuppression (1,12,15). Schlink *et al.* (16) reported that CY is not effective in depleting all the B cells, and a high dose of CY with bursectomy is required for ablating B cells from the immune system.

The objective of this study was to better characterize and describe the procedure by which CY induces B-cell ablation when administered *in ovo*. Such a model may be useful for deriving birds devoid of B cells to determine the protective components of the immune response *in vivo* against avian pathogens. The procedure was accomplished by treating chick embryos with CY during incubation. After hatching, blood samples were collected and evaluated by flow cytometry, blastogenesis response to B- and T-cell mitogens, and specific antibody response to a Newcastle disease vaccine.

MATERIALS AND METHODS

Eggs. Specific-pathogen-free (SPF) white leghorn eggs were purchased (Hy-Vac Co., Gowrie, IA). Birds that hatched were reared on wire-floored cages for 3 wk. Feed and water were provided *ad libitum*. The birds were also provided with antibiotics (aureomycin, chlortetracycline hydrochloride), 3 g/liter in water, until they were 2 wk of age.

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

RPMI 1640 supplemented with 25 mM HEPES (*N*-[2-hydroxyethyl]peperazine-*N'*-[2-ethanesulfo nic acid]) and L glutamine (Sigma Chemical Co., St.

Louis, MO), penicillin (200 μg/ml), and streptomycin (200 μg/ml) was used for washing and resuspending cells, diluting the mitogens and antigens, 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 concentration of 50 μg/ml for whole blood. 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 concentration of 1 μg/ml for whole blood response. An MTT (3-[4,5-dimethylthiazole-2-yl],2-5-diphenyltetrazolium bromide) (Sigma) solution 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.

CY treatment. In the first trial, 164 eggs were injected with CY at 16, 17, and 18 days of embryonic development. In addition, 30 eggs were injected with phosphate-buffered saline (PBS). This procedure was done by candling eggs to identify the air sac of the embryo. The area of the shell above the air sac was disinfected with 3% tincture of iodine. A drill was used to make a small hole in the eggshell. An aqueous solution of CY (0.1 ml, 20 mg/ml) was injected below the air cell membrane by inserting a 25-gauge, ⅝-inch (16-mm) needle attached to a syringe. The hole was then sealed with transparent tape and the eggs were returned to the incubator. The same procedure was used to inject the control eggs with CMF-PBS at day 16 of incubation and on the two succeeding days. The same procedure was repeated in the second trial in which 144 eggs were injected with CY and 50 eggs were injected with CMF-PBS.

Virus. The lentogenic type B1, strain B1, of Newcastle disease virus (NDV) (Intervet America Inc., Millsboro, DE) was propagated in 9-day-old embryonated SPF eggs. Embryonated eggs were inoculated by the chorioallantoic route and incubated for 5 days at 37 C. The allantoic fluid was harvested and clarified by centrifugation at 3000 × *g* for 30 min. The virus was purified and concentrated from the allantoic fluids (see below). The purified virus was then inactivated by exposure to ultraviolet light for 40 min and evaluated to assure loss of infectivity in embryonic eggs. The inactivated NDV was used as an inoculum at 40 μg per bird.

Virus propagation and purification. NDV purification was based on the method of Alexander (2,3). After NDV propagation (see above), the allantoic fluid was harvested and 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 ethylenediaminetetraesetic acid, pH 7.4. After centrifugation for 2 hr at 50,000 × *g*, a virus band was observed at the sucrose gradient interface. The virus band was collected and pelleted at 50,000 × *g* for 2 hr. The pellet was resuspended in PBS. The purified virus was assayed for total protein concentration by Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA), and the purity was assessed by Coomassie blue-stained polyacrylamide gel electrophoresis.

Whole blood. Whole blood was collected from the wing vein of the chickens with a syringe containing heparin (20 units/ml). The whole blood was used for the colorimetric blastogenesis microassay.

Colorimetric blastogenesis assay. The procedure for the blastogenesis microassay was performed similarly to that previously described (11). Briefly, the assay was carried out in 96-well flat-bottomed tissue culture plates (Corning Laboratory Sciences Co., Corning, NY). Two hundred microliters of RPMI 1640 containing Con A 50 μg/ml or LPS 1 μg/ml or media without mitogens (control wells) was dispensed in each well. Ten microliters of whole blood was added in each well. The plate was incubated at 37 C for 93 hr in a humid atmosphere of 5% CO_2. At 93 hr of the 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 at room temperature. The supernatant was removed carefully, and 150 μl of a 10% saponin solution was dispensed into each well of the plate to lyse the cells. The plate was shaken for 20 min on a plate shaker (mini-orbital shaker; Bellco Biotechnology, Vineland, NJ), and the cells were thoroughly resuspended by a multiple pipetting with a micropipetter. The plate was centrifuged at 1000 × *g* for 10 min, and the supernatant was carefully removed. One hundred seventy-five microliters of 1 N HCl-isopropanol was added to each well to dissolve the formazan crystals, and the plate was shaken, resuspended, and centrifuged as above. One hundred microliters of supernatant was transferred to the corresponding wells of a new 96-well plate. The absorbance of each well was measured with a microliter enzyme-linked immunosorbent assay reader (Model EL310; BIO-TEK Instruments, Inc., Winooski, VT) at a wavelength of 550 nm.

Flow cytometric analysis. An anti-CD3 mouse monoclonal antibody (MAb) (Southern Biotechnology Associates, Inc., Birmingham, AL) was used at 1:200 dilution. Anti-mouse immunoglobulin G (IgG)

(Fc specific) conjugated to fluorescein isothiocyanate (FITC) (Sigma) was used at 1:500 dilution. Anti-mouse immunoglobulin M (IgM) polyclonal antibody (Fc specific) conjugated to FITC (Nordic Immunological Laboratories, Drawer, Capistrano Beach, CA) was used at a concentration of 1:200. The single color staining method was used to stain T and B lymphocytes as described by Chan *et al.* (5). Briefly, 3 ml of blood was collected from each chicken by venipuncture in a syringe containing 20 units/ml of heparin. The blood was diluted 1:1 with CMF-PBS, layered on Lymphoprep™ (Accurate Chemical & Scientific Corporation, 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 CMF-PBS. 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 CMF-PBS, 200 µl of purified lymphocytes was added into each of three 1.5-ml microfuge tubes. The first tube contained 200 µl of diluted anti-CD3 MAb, the second tube contained 200 µl anti-mouse IgM conjugate FITC, and the third tube contained CMF-PBS (negative control). The three tubes were incubated for 30 min on ice. After incubation, 200 µl of CMF-PBS was added to each tube, and then the tubes were centrifuged at 500 × *g* for 5 min. The supernatant was discarded, and 200 µl of anti-mouse IgG-conjugated FITC was added only to the tubes containing anti-CD3 and reincubated for 30 min on ice. After the incubation period, these tubes were rinsed as before. The remaining cells were rediluted to the final volume of 200 µl in CMF-PBS. The staining percentages for each sample preparation were determined by flow cytometric analysis on an EPICS 752 flow cytometer (Coulter Corp., Hialeah, FL).

Hemagglutination inhibition (HI) test. The HI test was performed as described (4) with 10 hemagglutination units of antigen and 0.05% suspension of turkey erythrocytes.

Experimental design. At 2 wk of age, all *in ovo* CY-treated and CY-nontreated birds were bled, and the MTT blastogenesis microassay was performed with B- and T-cell mitogens. Statistical analysis with a *t*-test was performed to select those birds that had significant T-cell response and no significant B-cell response. At 3 wk of age, CY-treated birds that had significant Con A (T cell) response and no significant LPS (B cell) response were divided into two groups. The birds in group 1 (group CY-NDV) were each vaccinated subcutaneously with approximately 40 µg/bird ultraviolet (UV)-inactivated NDV (UV-NDV). The birds in group 2 (group CY-PBS) served as unvaccinated control birds and were injected with PBS. Similarly, the CY-untreated birds that were Con A (T cell) and LPS (B cell) responsive were divided into two groups (groups 3 and 4). Group 3 (group C-NDV) birds were vaccinated subcutaneously with approximately 40 µg/bird of UV-NDV. The birds in group 4 (group C-PBS) were injected with PBS and served as untreated, unvaccinated controls. The chickens were boostered at 5 wk of age, and blood samples were collected weekly. The MTT blastogenesis microassay and flow cytometric analysis were performed 1 wk after vaccination and 1 wk after the booster. The samples used for flow cytometric analysis were pooled samples from four chickens within each group. The antibody response to NDV for each bird was evaluated at 8, 14, and 24 days by HI.

Two trials were conducted. In the first trial, 10 birds (CY-NDV) were used from group 1 and five chickens from groups 2, 3, and 4. In the second trial, 12 chickens were used per group.

Statistical evaluation. To study the mitogenic response of each bird to B- and T-cell mitogens, analysis of variance (ANOVA) and *t*-tests were performed. A comparison was made between the absorbencies from four wells of cultured lymphocytes response without mitogen and the absorbencies from four wells of cultured lymphocytes responding to each mitogen (LPS, Con A) for each bird. Differences were determined as highly significant ($P < 0.01$), significant ($P < 0.05$), or not significant ($P > 0.05$). The average body weight in the CY-treated group was compared with that of the nontreated group by ANOVA. The percentages of B-cell and T-cell staining were compared between CY-treated birds and nontreated birds by ANOVA.

RESULTS

Effect of *in ovo* CY treatment on hatchability, mortality, and body weight. CY treatment *in ovo* resulted in decreased hatchability and increased mortality, as determined at 2 wk posthatch. *In ovo* CY treatment also significantly depressed body weight at 3 wk of age compared with CY-nontreated control birds (see Table 1).

Whole blood blastogenesis microassay. Fig. 1 depicts the responses of peripheral blood lymphocytes (PBL) of 1-day-old birds hatched from CY-treated eggs and CY-nontreated eggs to Con A and LPS. The results show that reduced responses to both T- and B-cell mitogens were observed in all of the CY-treated birds. Significant PBL mitogenic responses in both T- and B-cell mitogens were observed in all CY-nontreated birds. Table 2 shows the responses of 2-, 4-, and 6-wk-old birds to Con A and LPS. Approximately 50% of the 2-wk-old birds

Table 1. The effect of *in ovo* cyclophosphamide treatment on hatchability, mortality, and body weight.

Trial	Treatment	Hatchability	Mortality[A]	Body weight[B] (g)
1	CY[C]	106/164 (64%)	13/106 (12.3%)	ND[D]
	C[E]	30/30 (100%)	0/30 (0%)	ND
2	CY	90/144 (62.5%)	5/90 (3.3%)	142.07*
	C	48/50 (96%)	0/48 (0%)	197.400

[A] Cumulative mortality at 2 wk of age.
[B] Average body weight of group at 3 wk of age.
[C] Birds hatched from eggs injected *in ovo* with cyclophosphamide.
[D] ND = not done.
[E] Birds hatched from eggs injected *in ovo* with PBS.
* Statistically significant difference from control group ($P < 0.005$).

were T-cell positive and B-cell negative at 2 wk of age. A number of birds that had T-cell responses but not B-cell responses were retained in the study for continued monitoring of the B-cell response, whereas those birds responding to Con A and LPS were eliminated from the study.

The blastogenesis results at 4 wk of age indicated that all birds responded to Con A and 1 of 15 (6%) and 2 of 24 (8%) of the CY-treated birds responded to LPS in the first and second trials, respectively. At 6 wk of age, all the birds continued to respond to Con A, and three of the CY-treated birds responded to LPS, with a total of 4 of 15 birds responding in the first trial. Similarly, in the second trial, two birds responded to LPS by 4 wk and an additional three birds responded by 6 wk, yielding a total of 5 of 24 birds responding at 6 wk.

Antibody response to Newcastle disease vaccine. The results of the NDV vaccination are shown in Table 3. Chickens hatched from eggs treated with CY and vaccinated with UV-NDV did not produce detectable antibodies at 8 and 14 days postvaccination. However, 3 of 10 birds in trial 1 and 3 of 12 birds in trial 2 from the CY-treated group vaccinated with UV-NDV developed detectable antibodies during the first week postbooster. Antibody titers were detected in some of the CY-nontreated birds that were vaccinated with UV-NDV by 2 wk postvaccination, and all produced antibody titers after boosters.

Effect of CY on T- and B-cell populations in PBL. Table 4 shows the results from trials 1 and 2. In the first trial, the flow cytometric analysis indicated that the percentage of lymphocytes expressing CD3 in the CY-treated group was significantly less than in nontreated groups. No significant differences were found in the percentages of CD3-positive cells in either the CY-treated birds or the CY-nontreated birds in the second trial.

Significant differences were found in the percentages of IgM-positive cells between CY-treated birds and CY-nontreated birds. The staining percentages derived from CY-nontreated birds were higher than those from CY-treated birds.

DISCUSSION

CY treatment has been used as a means of abrogating the humoral immune response in order to determine the role of T and B cells in protective responses to infectious pathogens (9,12,13,15). CY injected during embryonic development has been reported to destroy both B and T lymphocytes. Subsequently, T cells were found to regenerate and repopulate lymphoid tissue in the majority of the treated chickens by 2 wk of age. However, B-cell regeneration remained suppressed for longer periods (7,14). The results of this study corroborate the effect of CY treatment reported in previous studies (7,14). Similar patterns of immunosuppressive effects induced by CY treatment were observed, whereby, after hatch, a severe lymphoid depletion of B and T cells was evident in the PBL of CY-treated birds, and functional recovery of T lymphocytes was observed in the majority of CY-treated birds by 2 wk of age. However, in the present study, the immunosuppressive effect of CY on B cells was not detected in all CY-treated birds. Additionally, the recovery of antibody-producing cells may have occurred earlier than 2 wk of age. A plausible reason for these disparities between

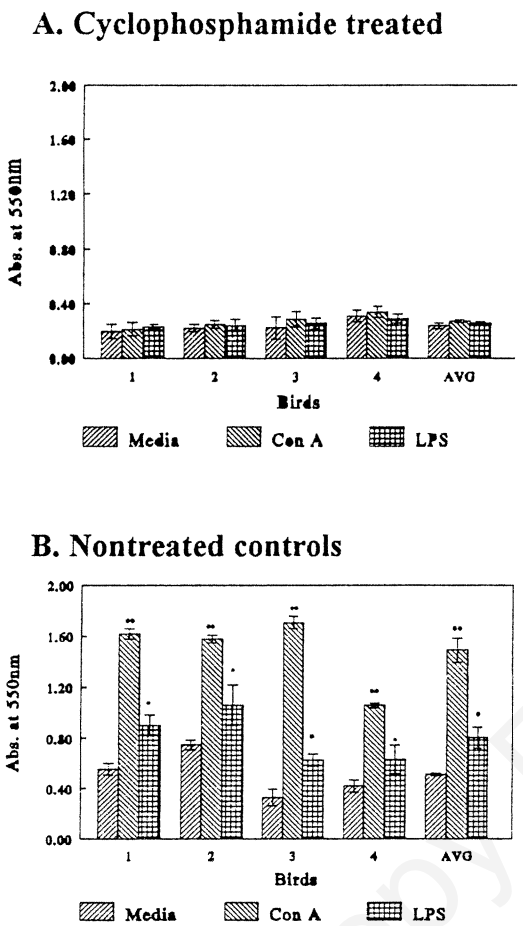

Fig. 1. Mitogenic response of peripheral blood lymphocytes to Con A and LPS (PBL) from four 1-day-old birds hatched from either CY-treated eggs (A) or nontreated control eggs (B). The averaged response of all four birds is also provided. The bars (of the individual birds) represent the average absorbencies from four replicates with the error bars indicating the standard deviation. A single asterisk (*) denotes a significant difference ($P < 0.05$) and two asterisks (**) denotes a highly significant difference ($P < 0.01$) in the mitogenic response when compared with the control treatment (no Con A or no LPS).

the studies may have been the route of CY injection into the eggs. Eskola and Toivanen (7) used intravenous injections of CY at 16–18 days of embryonic development to ensure a uniform distribution of the drug throughout the embryo. However, injecting CY by this method reduced hatchability and dramatically increased posthatch mortality, and, for those few birds that survived, there was a failure to fully regenerate T cells. Lymphocyte responses to phytohemagglutinin and Con A were significantly reduced at 44 days of age, which may have been because of the severe damage of the epithelial component of the thymus induced by the intravenous injection of CY. However, injecting CY below the air cell membrane, as described in this study, was found to be more convenient, time efficient, applicable for larger quantities of eggs, and yielded a substantially lower mortality rate when compared with the intravenous method.

The toxic effect of the CY treatment on hatchability, mortality, and body weight has been reported (1,6,7,14,15). Schlink *et al.* (16) found that high doses of CY administration destroyed both the B and T lymphocytes for long periods of time and increased the mortality rate. Low doses of CY, however, reduced mortality but were not sufficient to permanently affect the morphology or immunocompetency of the lymphoid organs of the bird (15). Other factors, such as strains of chickens, environmental factors, and the origin of CY, may also influence the immunosuppresive effect of CY. These observations emphasize the importance of determining an optimal dose of CY because considerable variability exists in the results reported among various investigations. The optimum dose in this study was determined by altering the concentration of CY and evaluating its effect on hatchability and 2-wk mortality (data not shown).

The flow cytometric analysis indicated a significant reduction of B cells in CY-treated birds as compared with the CY-nontreated birds. The percentage of anti-IgM+–stained cells in the CY-treated birds was within the background level of the assay. Therefore, although a low percentage of anti-IgM+–stained cells was found, this percentage was within the experimental error of the assay and was interpreted as being indicative of total B-cell ablation. The effect of CY treatment on the T cells was also demonstrated in CY-treated groups in the first trial. The percentage of T cells in CY-treated birds that were identified by CD3 markers was found to be approximately 13% less than that of CY-nontreated birds. However, these differences were not detected in the second trial in which the percentage of CD3 was not significantly different from that of the control group. The difference between the results in the first

Table 2. Mitogenic responses of PBL of 2-, 4-, and 6-wk-old birds hatched from CY-treated eggs and CY-nontreated eggs to Con A and LPS.

Trial	Week	No.	Treatment[A]	Con A(+)[B]	LPS(+)[C]	Con A(+), LPS(−)[D]
1	2	92	CY	75/89 (84%)	36/89 (40%)	46/89 (51%)
		10	C	10/10 (100%)	10/10 (100%)	0/10 (0%)
	4	15	CY	15/15 (100%)	1/15 (6%)	14/15 (93%)
		10	C	10/10 (100%)	9/10 (90%)	1/10 (10%)
	6	15	CY	15/15 (100%)	4/15 (26%)	11/15 (73%)
		10	C	10/10 (100%)	8/10 (80%)	2/10 (20%)
2	2	85	CY	75/85 (88%)	27/85 (31%)	48/85 (56%)
		36	C	36/36 (100%)	36/36 (100%)	0/36 (0%)
	4	24	CY	24/24 (100%)	2/24 (8%)	22/24 (91%)
		24	C	24/24 (100%)	24/24 (100%)	0/24 (0%)
	6	24	CY	24/24 (100%)	5/24 (20%)	19/24 (79%)
		24	C	24/24 (100%)	24/24 (100%)	0/24 (0%)

[A] CY = birds injected *in ovo* with cyclophosphamide; C = birds injected *in ovo* with PBS.
[B] Number of birds that had a statistically significant response to Con A ($P < 0.05$).
[C] Number of birds that had a statistically significant response to LPS ($P < 0.05$).
[D] Number of birds that had a statistically significant response to Con A but not to LPS ($P < 0.05$).

and second trials may have been because of the variation among the samples that were analyzed. The total of the percentage of anti-IgM+ cells plus the percentage of CD3 cells should approximate 100%. This is noted with the control birds in both trials. However, the total percentage of stained lymphocytes in the CY-treated birds fell short of approximating 100%. This inconsistency was unexpected and cannot be adequately explained by the data or by the technicalities of the assay. However, several possibilities may explain this inconsistency. First, a percentage of cells may not have stained because they were mononuclear cells that do not display IgM+ or CD3 as surface markers. Second, the CD3- or IgM+-bearing cells may have been in a precursor state and thus not at a stage of maturation that would allow staining. Finally, the CY may have had some effect (i.e., cytotoxic effect) on the lymphocytes, whereby some lymphocytes were not expressing, or had lost the ability to express, surface markers.

The immunosuppressive effect of the CY treatment on the antibody response to NDV was in agreement with the birds' responses to LPS mitogen. Only those birds that had signif-

Table 3. Antibody response to UV-NDV vaccination from CY-treated and CY-nontreated birds.

		Postvaccination[B]		Postbooster
Trial	Treatment[A]	8 days	14 days	10 days
1	CY-NDV	0/10	0/10	3/10 (2.37)[C]
	CY-PBS	0/5	0/5	0/5
	C-NDV	0/5	3/5 (3)[C]	5/5 (5.4)
	C-PBS	0/5	0/5	0/5
2	CY-NDV	0/12	0/12	3/12 (2.6)
	CY-PBS	0/12	0/12	0/12
	C-NDV	0/12	4/12 (3)	12/12 (6.2)
	C-PBS	0/12	0/12	0/12

[A] CY-NDV = CY-treated birds vaccinated with NDV; CY-PBS = CY-treated birds injected with PBS; C-NDV = CY-nontreated birds vaccinated with NDV; C-PBS = CY-nontreated birds injected with PBS.
[B] Number of birds having HI titer > 1:2.
[C] Numbers in parentheses indicate the geometric mean titer of responding birds expressed as reciprocal $\log_2$.

Table 4. Effect of *in ovo* CY treatment on the percentages of B and T lymphocytes from the peripheral blood as determined by flow cytometric analysis.

Trial	Week	Treatment[A]	% Staining[B]	
			Anti-IgM[C]	Anti-CD3[D]
1	4	CY	1.0*	66.7*
		C	8.2	85.9
	6	CY	1.3*	70*
		C	17.9	83.2
2	4	CY	1.35*	73.1
		C	13.5	78.15
	6	CY	0.8*	85.95
		C	9.5	83.05

[A] CY = cyclophosphamide-treated birds; C = cyclophosphamide-nontreated birds.
[B] Asterisk indicates the value is significantly different from the control group ($P < 0.05$).
[C] Percentage of staining B lymphocytes with anti-IgM.
[D] Percentage of staining T lymphocytes with anti-CD3.

icant responses to LPS at 6 wk of age developed detectable specific NDV antibodies. The fact that a few of the 2-wk-old T-cell–positive/B-cell–negative birds responded at 4 and 6 wk reveals that the immunosuppressive effect of CY on the B-cell system is transient and functional recovery of antibody-producing cells could occur at any time, thus demonstrating the need for continuous monitoring. This might be achieved by employing a technique that would enable monitoring the humoral immune response in the CY-treated birds. In this regard, the suppressive effect of CY might be evaluated through flow cytometric analysis, blastogenesis response to B-cell mitogens, and specific antibody response to pathogens. Those birds that demonstrate a functional and/or morphologic recovery of B cells should be identified and noted.

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