INFECTION AND IMMUNITY, Nov. 1975, p. 1058-1064

Vol. 12, No. 5
Printed in U.S.A.

Effect of Cyclophosphamide on the Response of Chickens to a Virulent Strain of Marek's Disease Virus

VALI KERMANI-ARAB,[1]* T. MOLL, B. R. CHO, WILLIAM C. DAVIS, AND YUE-SHOUNG LU

Departments of Veterinary Microbiology and Veterinary Pathology, Washington State University, Pullman, Washington 99163

Received for publication 10 June 1975

The effect of cyclophosphamide on the pathogenesis of Marek's disease was examined in a line of chickens which is relatively resistant to Marek's disease. The injection of cyclophosphamide into newly hatched chickens delayed and reduced viremia and also reduced the development of Marek's disease lesions until 2 weeks after exposure to Marek's disease virus. The data indicate that a population of T cells susceptible to infection with virus and possibly viral transformation is affected by cyclophosphamide.

Marek's disease (MD), a lymphoproliferative disease of chickens, is caused by a group B herpesvirus (MDV) (6, 23, 40). Attenuated MDV and turkey herpesvirus vaccines have been used and proven to be effective in preventing the development of MD tumors (31). However, their mechanism of action is unclear. Vaccinated birds develop viremia with vaccine virus as well as virulent virus when exposed. Pretreatment of newly hatched MD-susceptible chicks with cyclophosphamide (CP) decreases viremia, reduces the incidence of disease, and abolishes the protective effect of vacine (30; Kermani-Arab et al., unpublished data). The suppressive effect of CP is transient on T lymphocytes but prolonged on the B lymphocytes (20). Polak and Turk (28) suggest that CP also may affect a short-lived, rapidly proliferating suppressor T lymphocyte in the same manner as B lymphocytes.

Purchase and Sharma (30) have suggested that the bursa-dependent lymphoid system may play a role in protection by vaccine virus. Studies by Biggs et al. (2) have indicated that the level of superinfection with virulent virus is related to the titer of vaccine virus in blood. These investigators have proposed virus interference as the mechanism involved in vaccine protection.

The purpose of the present investigation was to examine the effect of CP on the response of a relatively resistant chicken to a virulent strain of MDV and attempt to ascertain the role of CP in the alteration of vaccine protection. The parameters studied included pathogenesis of lesion formation, proliferation of virus, immunoglobulin and specific antibody synthesis, and T cell response to concanavalin A (Con A).

[1] Present address: Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, Ore. 97201.

MATERIALS AND METHODS

Experimental chickens. Male birds from a relatively MD-resistant line of feather-sexed commercial broiler breeder (H & N strain P) chickens were used (15), and all chickens were kept in Horsfall-Bauer isolators (14).

CP treatment. Each bird in groups 3 and 6 was given a total of 18 mg of CP (Mead Johnson Laboratories, Evansville, Ind.) intramuscularly (4.5 mg/0.25 ml daily per chick the first 4 days of life), and each bird in groups 2 and 5 was injected with a total of 8 mg of CP (2 mg/0.25 ml daily per chick the first 4 days of life). The birds in groups 1 and 4 received no treatment. Mortality of chicks shortly after CP treatment was interpreted as due to toxic effects of CP.

Virus exposure. A highly virulent strain of MDV (MDV/Id-1) causing acute MD (15a; 35) was grown and plaque purified in chicken embryo fibroblast cell cultures (4). The cell-associated stock virus was stored in liquid nitrogen until used (41).

A total of 1,500 plaque-forming untis (PFU) of MDV/Id-1 was inoculated subcutaneously into anti-MDV antibody-free birds (groups 1, 2, and 3) at 3 weeks of age. Virus control groups (4, 5, and 6) were kept unexposed throughout the experiment.

Sheep erythrocyte immunization. Five to ten birds from each group, free of anti-sheep erythrocyte (SRBC) hemagglutinin 21 days after hatching, were inoculated with 10^9 SRBC intramuscularly each week for 8 weeks.

Each week after exposure to MDV five to ten birds from each experimental group were bled by cardiac puncture and sacrificed. These birds were examined for viremia, MD pathology, spleen cell blastogenic response to Con A, levels of immunoglobulin (Ig)M, IgY, and IgA, antibody response to SRBC, and anti-MDV antibody production.

Examination of MDV viremia, MD lesion development, and blastogenic response of spleen cells to Con A. The viremia assay was conducted as de-

scribed by Cho and Kenzy (5). The virus titer, expressed as PFU, was the average number of plaques in two replica dishes (60 by 15 mm) of chicken embryo fibroblast monolayers inoculated with 0.1 ml of heparinized blood.

Pieces of peripheral nerves (sciatic, brachial, and vagus) and gonads from each bird were processed for histological examination (hemotoxylin and eosin stain). The MD nerve lesions were classified as A, B, and C according to Payne and Biggs (27). The size of spleen and bursa were measured and the absence or presence of germinal centers in spleens (10 microscopic fields × 100) was noted. The depletion of lymphoid cells in 50 bursa follicles was scored as 4+ ≦ 100%; 3+ ≦ 75%; 2+ ≦ 50%; and 1+ ≦ 25% loss of B cells. The blastogenic response of spleen cells to Con A was examined by the method of Lu and Lapen (21). The stimulation index was taken as the ratio in counts per minute of the mitogen-stimulated to the nonstimulated cultures.

Tests for antibody synthesis. For determination of levels of serum IgM, IgY, and IgA, monospecific anti-chicken immunoglobulins were prepared or obtained in the following manner. The maternal IgY was extracted from chicken egg yolks by cold chloroform by the method described previously (1, 42). IgY was purified by diethylaminoethyl-cellulose (Sigma, St. Louis, Mo.) chromatography followed by two Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N.J.) gel filtration (17, 19). Purified IgY was reduced with dithiothreitol (Cleveland reagent, Calbiochem, Los Angeles, Calif.) at a molar ratio of dithiothreitol:IgY = 100:1 in 0.15 M tris(hydroxymethyl)aminomethane-hydrochloride, 0.15 M NaCl, 0.002 M ethylenediaminetetraacetic acid, pH 8.0 (7, 10). Subsequently, the reduced immunoglobulin chains were alkylated with iodoacetamide (K and K Laboratories, Plainview, N.Y.) and then dialyzed against 1 M propionic acid. The reduced and alkylated chains were separated by column chromatography using Sephadex G-100 column (2.5 by 90 cm) equilibrated with 1 M propionic acid. The fraction containing H chains was then passed through the column twice. The H chain fraction obtained was concentrated to 3 mg/ml and checked for purity by the agar gel diffusion and immunoelectrophoresis tests. The protein (3 mg/ml) was emulsified and injected subcutaneously into a rabbit for 3 successive weeks (1 mg per injection) and bled 1 week after the last inoculation to obtain anti-IgY. Monospecific rabbit anti-chicken IgM and goat anti-chicken IgA were provided by G. A. Leslie (Department of Microbiology and Immunology, University of Oregon Medical School, Portland) to determine the level of antibodies in serum. Total serum levels of IgM, IgY, and IgA were measured by radial immunodiffusion (9, 18).

The response to SRBC was determined by using a microhemagglutination technique. The $\log_2$ of the highest dilution with a central clear zone of the settled cells was reported as the final hemagglutination titer.

Precipitins for MDV-associated antigens were determined by the agar-gel precipitin test (12, 24).

Monospecific rabbit anti-chicken IgM and IgY were conjugated with fluorescein isothiocyanate at the ratio of 40 μg of protein/1 μg of fluorescein isothiocyanate and used in the indirect fluorescent antibody technique to detect and measure the titer of IgM and IgY anti-MDV antibodies (37).

Statistical analysis. The rank test (39), Student's *t* test (38), and contingency tables (22) were employed.

RESULTS

Toxic effect of CP on newly hatched chickens. The total incidence of toxic mortality among 18- and 8-mg CP-treated chicks by day 10 after hatching was 65 and 29%, respectively. The total nonspecific mortality for untreated chicks was 3% during the first 10 days of life.

Immunosuppressive effect of CP on B lymphocytes. Examination of the bursae and spleens from birds treated with 18 mg of CP showed rudimentary, undeveloped bursa follicles with interfollicular fibrosis and atrophic spleens without germinal centers. This histological status persisted throughout the experimental period. Similar bursa and spleen tissue alterations were observed in 8-mg CP-treated birds until 7 weeks of age. Subsequently, the bursae and spleens developed the normal histology.

The determination of serum IgM, IgY, and IgA levels by radial immunodiffusion (Fig. 1) showed that all 18-mg CP-treated chickens remained agammaglobulinemic or severely hypogammaglobulinemic (<0.1 mg/ml) during the period of the study. These birds also were unable to produce antibody to SRBC or MDV. A small number of 8-mg CP-treated birds were agammaglobulinemic during the first 2 weeks after treatment, but the majority of the birds in these groups exhibited significantly higher ($P < 0.05$) levels of serum IgY and IgA than birds receiving 18 mg of CP but lower ($P < 0.05$) than untreated birds throughout the experimental period. However, a gradual increase of serum IgY and IgA was noted in birds receiving 8 mg of CP after 7 weeks of age. The serum IgM and anti-SRBC hemagglutinin levels increased rapidly and became markedly higher ($P < 0.05$) in birds receiving 8 mg of CP than in control birds after 7 weeks of age. The anti-MDV antibody levels in non-CP-treated MDV exposed birds were significantly higher ($P < 0.05$) than in MDV-exposed birds receiving 8 mg of CP. All birds not exposed to MDV remained free of anti-MDV antibodies during the experimental period.

Effect of CP on viremia. As shown in Table 1, viremia was detected in all five birds (range 10 to 115 PFU) from the non-CP-treated MDV-

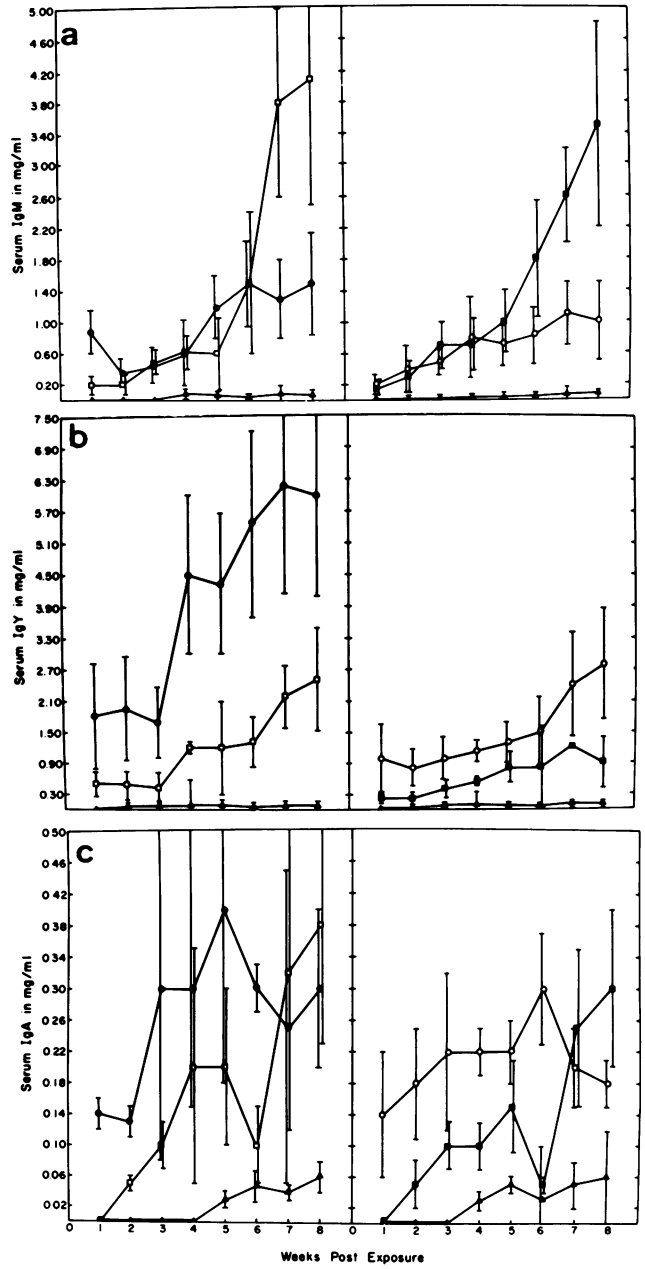

FIG. 1. (*a, b, and c*) *Comparison of the levels of immunoglobulins IgM, IgY and IgA in untreated chickens and chickens treated with CP with or without exposure to MDV. Each point is the mean of 5 to 10 birds. The vertical bars represent the standard deviation of the mean. Symbols: MDV-exposed chickens:* (●), *non-CP treated (group 1);* (□), *8-mg CP treated (group 2);* (△), *18-mg CP treated (group 3). Unexposed chickens:* (○), *non-CP treated (group 4);* (■), *8-mg CP treated (group 5);* (▲), *18-mg CP treated (group 6).*

TABLE 1. *Comparison of the incidence and level of MDV/Id-1 viremia in CP-treated and nontreated commercial (H&N) chickens at various intervals postinoculation*

Group	CP (mg)	Weeks after virus inoculation							
		1	2	3	4	5	6	7	8
1		5/5[a] (25)[b]	10/10 (209)	2/10 (0)	1/10 (0)	0/8 (0)	1/10 (0)	0/5 (0)	0/5 (0)
2	8	0/5 (0)	5/10 (20)	1/10 (0)	5/10 (0)	1/10 (0)	1/10 (0)	4/5 (6)	3/5 (4)
3	18	0/5 (0)	3/10 (0)	0/10 (0)	3/10 (0)	0/10 (0)	1/10 (0)	0/5 (0)	0/5 (0)

[a] Numerator is the number of MDV-positive birds; denominator is the number of birds examined.
[b] Median titer of virus.

infected group (group 1), whereas virus was not detected in the blood of any of the 8- or 18-mg CP-treated MDV inoculated birds (groups 2 and 3) 1 week after MDV exposure. At 2 weeks after exposure, 50 and 30% of the birds from groups 2 and 3, respectively, were viremic (range 10 to 240 and 1 to 21 PFU), but the extent of viremia was markedly reduced. Blood virus titers in all MDV-exposed birds were not significantly different at any time from 3 to 7 weeks postexposure. After 7 weeks, however, the incidence of viremia increased in birds receiving 8 mg of CP. Control birds not exposed to MDV were free of detectable MDV throughout the experimental period.

Effect of CP on MD lesions. Two weeks after exposure, most of the MDV-exposed non-CP-treated birds (group 1) had enlarged spleens. Some of the spleens from these birds showed small lymphoid-like tumors under the capsule. Spleens from birds in the MDV-exposed, 8- and 18-mg CP-treated groups (2 and 3) were generally small and free of any detectable tumors. Subsequently, MD lesions were detected in a few birds from group 2, whereas group 3 birds remained free of MD lesions until 5 weeks postexposure, and only two birds in this group developed typical MD lesions in the following weeks.

Microscopic examination of tissues from group 1 birds revealed lymphoid proliferative lesions in peripheral nerves and gonads early in the course of the disease (Table 2). Mild neural lesions (type C) were present in all five birds 1 week after exposure, whereas lesions were not observed in any group 2 and 3 birds at this time. At 2 weeks postexposure MD lesions were observed in all birds from group 1, most birds from group 2, and only a few birds in group 3. Group 3 birds exhibited a significantly lower ($P < 0.05$) incidence of neural lesions than group 1 chickens up to 4 weeks after exposure, but subsequently groups 1, 2, and 3 birds did not reveal an appreciable difference in an incidence of the neural lesions. The development of MD lesions in gonads followed a similar course, but only a few of the birds from each group (Table 2, groups 1 to 3) showed detectable lesions at each period of examination. The control birds (groups 4 to 6) remained free of any detectable MD lesions throughout the experiment.

Effect of CP on the blastogenic response of spleen cells to Con A. Spleen cells from individual birds from the various experimental groups showed a wide variation in their blastogenic response to Con A throughout the experimental period. The blastogenic response in most birds appeared lower at 4 weeks of age

TABLE 2. *Effect of CP on the development of MD lesions in relatively MD-resistant chickens (H&N)*

Weeks after virus inoculation	Total no. of birds examined	Types of nerve lesions[a]									Severity of MD lesions in gonads[b]									
		Group 1[c]			Group 2			Group 3			Group 1				Group 2				Group 3		
		A	B	C	A	B	C	A	B	C	1+	2+	3+	4+	1+	2+	3+	4+	1+	2+	3+	4+
1	5	0	0	5[d]	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
2	10	0	1	9	0	0	7	0	0	3	3	0	0	0	5	0	0	0	3	0	0	0
3	10	0	1	8	0	0	7	0	0	4	2	0	0	0	3	0	0	0	3	0	0	0
4	10	0	0	10	0	0	5	0	0	3	4	0	0	0	2	0	0	0	0	0	0	0
5	10	0	0	8	0	1	6	0	0	5	1	0	0	0	2	0	0	0	3	0	0	0
6	10	0	1	7	0	0	7	0	0	8	2	0	0	0	2	0	0	0	2	0	0	0
7	5	0	0	4	0	0	3	0	0	4	1	0	0	0	1	0	0	0	1	0	0	0
8	5	0	0	4	0	0	4	0	0	3	0	0	0	0	2	0	0	0	2	0	0	0

[a] Classified into types A, B, and C according to Payne and Biggs (27).
[b] Grading by the extent of mononuclear cell infiltration: 1+ ≦ 25%, 2+ ≦ 50%, 3+ ≦ 75%, and 4+ ≦ 100%.
[c] Groups of 1 to 3 chickens represent non-CP-treated birds and those injected with 8 and 18 mg of CP, respectively.
[d] Total number of birds with lesions.

than during the following periods. This may possibly be due to an inadequate number of birds tested or lack of complete maturation of Con A responding to T cells in the spleen at that age. A weekly comparison (Table 3) between groups 1 and 2, 1 and 3, 1 and 4, 2 and 5, and 3 and 6 birds indicated no significant difference in the blastogenic response. However, at the late stages of the experiment, the median response of cells from birds treated with 8 mg of CP and exposed to MDV was lower than that of cells from the other groups.

DISCUSSION

The data obtained from this study provide additional evidence of an immunosuppressive effect of high doses of CP on bursa-dependent immune functions (20, 30, 33, 43). Moderate doses of CP seem to exert a transient effect on B lymphocytes. The response of spleen cells to Con A which determines T-cell function (44) appears to be normal in chickens 4 weeks after treatment with high or moderate doses of CP. Treatment with CP was also effective in reducing MDV viremia and delaying the development of MD lesions.

The exact mechanisms involved in these changes after treatment with CP remain to be elucidated. The elimination of B cells as a possible controlling factor of the pathogenesis of MD is unlikely since MD lymphomas are considered to be mainly a T-cell population (11, 32). Furthermore, complete surgical bursectomy of 17-day-old embryos does not alter host resistance to MD in susceptible or resistant chickens (34). CP treatment of MD-susceptible chickens at the time of hatching has a severe effect on B cells; it reduces viremia, MD lesions, and mortality, and prolongs normal blastogenic response of spleen cells to Con A (30; Kermani-Arab et al., unpublished data). The effect of CP on T-cell regulation in MD is unknown. However, thymectomy of susceptible or resistant birds decreases or increases development of MD lymphoma, respectively. On the basis of these findings, a dual nature of thymus-dependent lymphoid cells, namely effector and targe cells, has been suggested (26). In the present study, treatment with CP markedly reduced viremia and delayed or decreased the formation of MD lesions. Thus, it is likely that high-dose CP has selectively prolonged the suppressive effect on the target T cells. The elimination of vaccine protection in CP-treated birds reported by Purchase and Sharma (30) may also be explained by the restriction of common target cells for vaccine virus as well as virulent MDV; this in turn results in the destruction, interference, or cell blocking phenomenon as described previously (2, 36).

It is tempting to speculate that the target T cells in susceptible chickens readily undergo transformation when associated with virulent MDV. Subsequently, these cells exert a suppressive effect on both effector T cells and antibody-forming cells. This is consistent with the depres-

TABLE 3. *Effect of CP on the blastogenic response of spleen cells from relatively MD-resistant chickens (H&N) exposed to MDV/Id-1 and Con A*

Group	Median and range of SI[a] at various weeks postinoculation							
	1	2	3	4	5	6	7	8
1	(5)[b]	(5)	(5)	(8)	(7)	(5)	(3)	(3)
	1.75	10.1	7.2	3.8	10.30	10.14	11.46	14.60
	0.50–6.06	0.80–38.60	1.60–13.75	0.49–14.7	0.75–26.92	1.28–122.90	0.67–22.5	1.60–30.5
2	(5)	(6)	(7)	(8)	(8)	(5)	(5)	
	3.70	13.60	3.75	3.22	9.3	1.80	1.77	NA[c]
	0.93–5.5	1.2–185	0.73–100	1.00–20.2	1.48–130.66	0.510–25	0.75–22	
3	(5)	(8)	(6)	(8)	(8)	(5)	(5)	(4)
	3.80	10.42	3.15	2.40	8.74	10.50	18.09	12.25
	0.80–5.7	1.50–86.3	1–12.50	1.00–70	2.8–210.44	1.02–40.03	1–105.55	0.9–18.8
4	(3)	(4)	(4)	(5)	(6)	(5)		(3)
	3.06	12.09	12.2	5.63	15.22	9.15	NA	28
	1.06–7.2	1.77–163.22	1.00–23.99	1.50–5.94	2.3–67.46	1.1–119.94		1.45–88
5	(3)	(5)	(4)	(4)	(6)	(5)	(3)	(3)
	3.20	10.23	9.9	10.47	10.75	14.55	19.02	22.10
	1.02–4.20	0.93–128	1.84–90.30	1.1–86.03	0.82–44.36	1.02–124.20	1.37–21.03	11.07–84.19
6	(3)	(5)	(5)	(5)	(5)	(5)	(3)	(4)
	3.71	7.30	7.03	9.31	17.38	17.02	18.36	14.33
	1.12–7.03	3.3–10.81	1.1–38	6.07–23.04	6.76–54.25	0.8–39.27	2.00–39.5	2.00–54.00

[a] SI, Stimulation index.
[b] Number of birds tested.
[c] NA, Not available.

sion of capacity to reject skin graft (25, 29) and loss of in vitro blastogenic activity, as well as suppression of antibody synthesis during the progression of MD (3, 8, 13, 16, 21, 29). Thus, this also may be a plausible explanation for the effect of CP on viremia and the development of MD lesions.

ACKNOWLEDGMENTS

This paper was supported by Public Health Service grants GM 00691, CA 13865, and FR 5465 from the National Institute of General Medical Sciences, the National Cancer Institute, and the Division of Research Facilities and Resources, respectively.

We thank Margaret Hinz, Stephani Moore, and Roberta Pritchard for their technical assistance, and Mary Estes for secretarial assistance.

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