INFECTION AND IMMUNITY, July 1977, p. 227-230
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Vol. 17, No. 1
Printed in U.S.A.

Suppressive Effect of Cyclophosphamide on the T-Cell System in Chickens

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Received for publication 4 February 1977

Neonatal administration of 16 mg of cyclophosphamide in inbred chickens resulted in a transient, but profound, deficiency in the in vitro proliferative response of spleen cells. Functional T-cell deficiency was accompanied by a marked morphological degeneration in the thymus and thymus-dependent areas in the spleen.

Cyclophosphamide (CY), a widely used antitumor drug, is metabolized in the liver and yields substances that exert toxic effects on certain cells (4). Because lymphocytes are very vulnerable to the cytotoxic effect of CY, the drug has also been used extensively in research in recent years as an immunosuppressive agent. Numerous studies have shown that CY is primarily a B-cell suppressant (12, 14, 19, 23). In chickens treated neonatally with CY, for example, immunoglobulin synthesis is severely inhibited, and the B-cell population in the major lymphoid organs virtually disappears (12, 13). Although CY-induced B-cell suppression in chickens has been well characterized, reports on the effect of CY on the T-cell system are conflicting. For instance, CY has been interpreted to have no effect (9, 12), a transient inhibitory effect (13, 17, 21), or a long-lasting inhibitory (6, 18) effect on T-cell functions. In mammalian models, the T-cell system has been generally considered spared by CY (11, 22-24), although under certain circumstance, T cells seem clearly affected (1-3, 8, 25, 27). Possibly, one of the underlying reasons for the conflicting result has been the variation in the experimental design, particularly the dose of the drug, the age and genetic background of the animals, the intervals between the administration of the drug and the assessment of the T-cell function, and the method of testing T-cell reactivity in vitro (15).

In an earlier study, Purchase and Sharma (16) noted that chickens treated with CY before vaccination with the herpesvirus of turkeys were not protected against Marek's disease. Because bursectomy alone did not influence vaccination (5), we postulated (28) that the inhibitory effect of CY may be mediated through a deficiency in the T-cell system.

This study was designed to examine, on a chronological basis, the effect of CY treatment on morphological and functional integrity of the T-cell system in chickens. The regimen of drug administration was consistent with that most frequently used to produce selective B-cell suppression. Our results clearly show a transient, but profound, T-cell deficiency in CY-treated chickens.

MATERIALS AND METHODS

In two similar experiments, chickens were progeny of a cross between inbred lines 15 and 7 (20). In experiment I, each of a group of chickens was given 4 mg of CY (Cytoxan, Mead Johnson Laboratories, Evansville, Ind.) intra-abdominally per day for the first 4 days after hatching. Other chickens were left as untreated controls. The two groups were housed in separate filtered-air, positive-pressure units throughout the experiment. At various intervals after the last injection of CY, chickens from CY-treated and untreated groups were randomly sampled and examined as follows. (i) Viable cell suspensions from individual or pooled spleens were cultured in vitro in Microtest II plates (Falcon) and examined for their response to phytohemmagglutinin (PHA) stimulation according to the general procedures described earlier (10); (ii) the thymus (all lobes on both sides of the neck), bursa, and spleen were excised, and wet weights of each organ were determined with an electronic balance (Mettler); and (iii) Formalin-fixed sections of thymus, bursa, and spleen were examined histologically.

Ten chickens of each CY-treated and untreated group were inoculated intravenously with sheep erythrocytes (10^9 cells) and *Brucella abortus* (10^{10} cells) at 21 and 28 days of age. Sera collected 7 days after the second injection were tested for antibodies against the injected antigens. Hemagglutinins against sheep erythocytes were assayed by a tube agglutination test (26), and antibodies against *B. abortus* were assayed by the plate agglutination test.

RESULTS

CY-treated chickens were severely deficient in antibody synthesis when tested 3 to 4 weeks after CY treatment. The mean $\log_2$ sheep erythrocyte agglutinin titer was 3.2 ± 0.25 in the treated group and 9.5 ± 0.27 in the untreated group. The respective $\log_2$ *B. abortus* antibody titers in treated and untreated groups were 3.7 ± 0.31 and 6.1 ± 0.29. These results indicated that the stock of CY used had the expected inhibitory effect on the B-cell system.

Table 1 gives the data from two experiments on the response of spleen cells from CY-treated and untreated chickens to PHA, a T-cell mitogen (7). The stimulation indexes (counts per minute of [^{3}H]thymidine incorporation in cells treated with PHA divided by counts per minute in cells without PHA) of CY-treated chickens were appreciably lower than indexes in the untreated controls during the first 7 to 9 days, thus suggesting a deficiency in responsive T cells. The differences in stimulation indexes between CY-treated and untreated groups were statistically significant at day 4 ($P < 0.05$, run test) and day 7 ($P < 0.01$) in experiment 1 and at day 5 ($P < 0.05$) and day 9 ($P < 0.01$) in experiment 2. These results indicated that the proliferative response of spleen cells in the CY-treated chickens was depressed during the initial stages after drug administration and that this function was restored to near normal levels within 1 to 2 weeks. Thus, in contrast to the persistent defect in the B-cell system, the defect in the proliferative response of T cells was transient and reversible.

TABLE 1. *Response of spleen cells to PHA stimulation in CY-treated and untreated chickens*

Days after last CY injection	Mean stimulation index[a] (± standard error of mean)	
	CY (16 mg)	No CY
Expt 1		
2	0.97 ± 0.11	5.31 ± 1.50
4	1.26 ± 0.19	5.07 ± 1.62[b]
7	3.72 ± 1.05	9.28 ± 0.91[c]
11	5.39 ± 0.69	6.55 ± 0.24
14	11.76 ± 0.67	12.85 ± 1.71
21	10.66 ± 2.49	11.00 ± 1.03
43	9.06 ± 0.60	8.76 ± 0.63
Expt 2		
2	0.65	21.14 ± 3.35
5	1.77 ± 0.62	27.00 ± 5.95[b]
9	7.60 ± 2.82	30.68 ± 4.95[c]
16	28.7 ± 7.32	35.70 ± 8.83
46	72.35 ± 9.35	90.40 ± 18.07

[a] Each mean represents the average of five individual spleen cell suspensions. At 2, 4, and 7 days in experiment 1 and at 2 and 5 days in experiment 2, 1 to 4 suspensions were tested, and each suspension was a pool of two to five spleens.

[b] Stimulation indexes of CY-treated and untreated chickens were significantly different ($P < 0.05$).

[c] Stimulation indexes of CY-treated and untreated chickens were significantly different ($P < 0.01$).

The morphological alterations in the thymus and in thymus-dependent areas of the spleen were also striking during the first few days after drug administration. During the initial stages, the thymus was almost completely depleted of thymocytes both in the cortical and in the medullary areas (Fig. 1), and thymus-dependent areas in the spleen lacked detectable lymphoid cells. Thymocyte depletion in the thymus was accompanied by an increase in the reticular cell framework. As shown in Fig. 2, thymic follicles became rapidly repopulated with thymocytes and attained normal appearance by day 11 after CY treatment. The degenerative changes in bursal morphology and the absence of germinal centers in the spleen were severe, as reported previously (13).

The morphological degeneration of lymphoid cells in various lymphoid organs of CY-treated chickens was also reflected in the mean wet weight of the organs (Fig. 3). The bursal weights in CY-treated chickens remained significantly lower than those of untreated chickens throughout the experiment ($P < 0.01$ at each interval). The thymus weight, on the other hand, which was significantly lower ($P < 0.01$) in CY-treated chickens at 2, 4, and 7 days, approached normal levels by 14 days after treatment. At 21 days, thymus of CY-treated chickens was significantly heavier than that of untreated chickens ($P < 0.01$). Similarly, spleen weights in the CY-treated chickens at 21 days had fully recovered to above-normal levels; however, the spleen weight in CY-treated chickens was significantly lower than that of untreated chickens at 2, 4, 7, 11, and 14 days ($P < 0.01$).

DISCUSSION

The above results clearly indicated that the T-cell mitogen responsiveness of spleen cell suspensions in CY-treated chickens was significantly reduced during the first 1 to 2 weeks of CY administration. The reduced responsiveness of T cells was accompanied by a marked morphological degeneration in the major lymphoid organs. The T-cell areas, namely, the thymus and the thymus-dependent areas of the spleen, showed apparent rapid regeneration, whereas the B-cell areas remained depleted

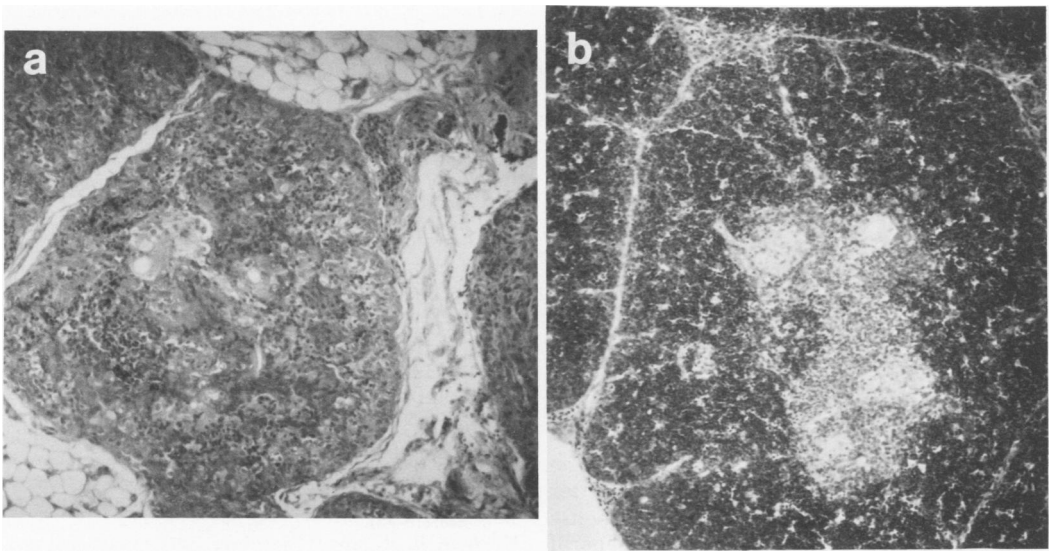

FIG. 1. *(a) Section of a thymus of an 8-day-old chicken. The thymus was removed 4 days after CY treatment. Note the depletion of thymocytes in the cortical and the medullary areas. (b) Section of a thymus of an untreated 8-day-old control chicken. ×129.*

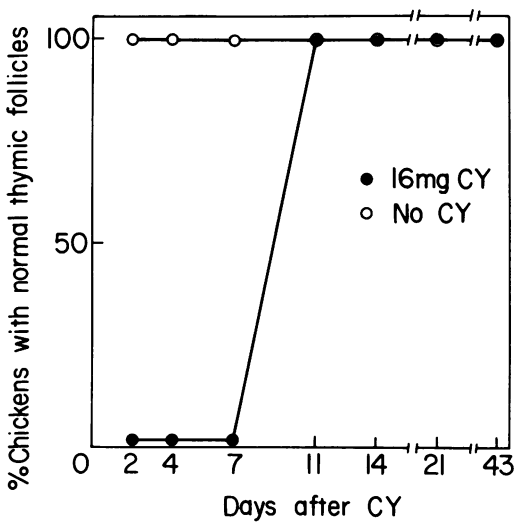

FIG. 2. *Effect of CY on the histological appearance of thymic follicles. At each interval, four to six chickens and at least eight thymus lobes per chicken were examined. If >80% of the follicles examined had no detectable degenerative change, the thymus was considered normal.*

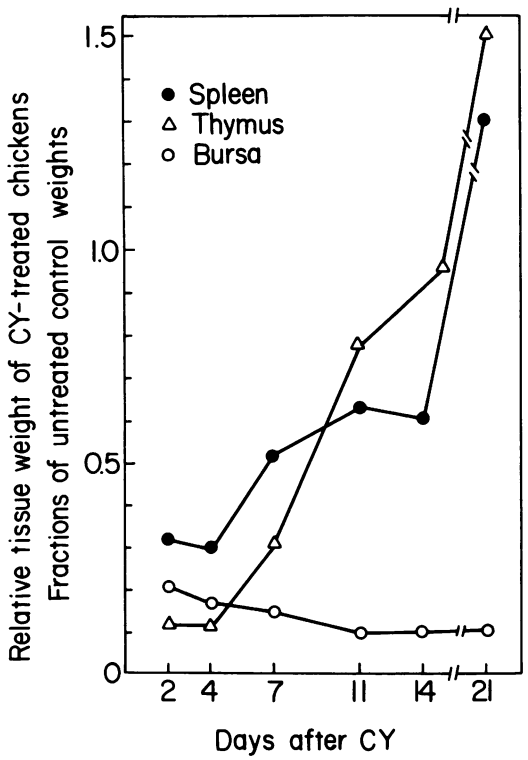

FIG. 3. *Relative weights of spleen, thymus, and bursa of chickens. Each point is an average of five chickens. The average weight of a given tissue of untreated chickens at each interval was considered as 1, and the weight in CY-treated chickens is expressed as a fraction of 1.*

throughout the observation period. The morphological repair in the T-cell areas was accompanied by a near-normal proliferative response of spleen cell suspensions to PHA stimulation. Thus, we conclude that in chickens, CY initially induces an overall cytotoxic effect on both B and T lymphoid cells. The T-cell system regenerates, presumably because some stem cells

of the T-cell lineage survive cytotoxicity, whereas cells of the B-cell system do not recover appreciably. The extent to which T-cell systems recover in CY-treated chickens may be influenced by several factors such as the initial dose of CY and the age and genetic strain of chickens. Others have found that graft-versus-host response and in vitro reactivity to mitogens in chickens treated with CY as embryos was depressed for up to 6 weeks of age (6, 18). Thus, some T-cell functions possibly may be persistently damaged by CY.

The inhibitory effect of CY on the T-cell system in our study and in others (6, 18, 21) should be kept in mind when this drug is used as a specific B-cell suppressant, particularly in studies on the effect of selective B-cell immunosuppression on disease response. If disease-causing agents are administered shortly after CY treatment, the effect on disease response should be viewed as being that of a general immunosuppression in both B- and T-cell systems rather than in the B-cell system alone.

ACKNOWLEDGMENTS

We wish to thank Charles Graham for statistical analyses and Barry Coulson and Evelyn Young for technical assistance.

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