CYCLOPHOSPHAMIDE-INDUCED IMMUNOLOGIC DEFICIENCY IN IMMATURE CHICKENS

F. Seto, J. D. Riddle, and W. G. Henderson

Department of Zoology, University of Oklahoma, Norman, Oklahoma

Varying degrees of immunological deficiency were observed during the first few weeks after hatching among chicks injected with cyclophosphamide as 17- to 18-day embryos or day-old chicks. Natural agglutinin levels were considerably below normal, onset of the hemagglutinin-forming potential was delayed, and growth of the immune capacity, as measured by hemagglutinin titers and antigen-sensitive units in the blood of immunized birds, was much suppressed. With regard to the three experimental measurements of immunosuppression, the magnitude and duration of the immunologic deficiency were, generally, dose-dependent. The nature of the immunosuppressive effect is discussed briefly.

The effects of radiation and immunosuppressive drugs on the immune response have been investigated extensively in young and adult mammals (1-8), but less attention has been directed to comparable kinetic studies with chickens and other non-mammalian vertebrates. Relatively little is known of the effects of cytotoxic agents on the ontogeny of the immune potential in embryos and newly hatched chicks (9-11). Our earlier studies indicate that X-irradiation of embryos suppresses the graft-versus-host (GVH) reaction elicited by grafted allogeneic spleen cells, and X-irradiation of baby chicks depresses their hemagglutinin-forming capacity (12, 13). Moreover, similar immunosuppressive effects were observed in embryos and baby chicks treated with the alkylating agent cyclophosphamide (13, 14).

In the course of an ontogenetic study of the immune potential of the chicken, we determined the optimum cyclophosphamide dose required to suppress the immune response of host embryos and baby chicks for use in the allogeneic *in vivo* transfer system (15). In the study reported here, the effects of different doses of cyclophosphamide on the development of (a) natural hemagglutinins, (b) immune hemagglutinin-forming capacity, and (c) antigen-sensitive unit-forming capacity were determined.

MATERIALS AND METHODS

Hybrid California gray x white Leghorn chicks, hatched from eggs obtained from a local hatchery, and F_2 chicks obtained from the hybrids in our own pens served equally well for the experiments. The chicks were housed in a Sears battery brooder the first 3 weeks and in developer cages later. Purina chicken feed and water were provided *ad libitum*.

Drug treatment

A saline solution of cyclophosphamide (Cytoxan, Mead Johnson and Co., Evansville, Indiana) was freshly prepared for each experiment and administered within an hour. A ½ ml tuberculin syringe fitted with a 30 gauge needle was used to inject specific amounts of the drug into the chorioallantoic vein of embryos through windows cut in the shell. The windows were sealed with transparent tape and the eggs were returned to the incubator. Groups of 17- to 18-day embryos were thus injected intravenously with 2, 3, 4, or 5 mg of cyclophosphamide (Cy) in 0.1 to 0.2 ml of solution per embryo.

Groups of day-old chicks received single intraperitoneal injections of 2, 4, or 8 mg of Cy in 0.5 ml of solution per bird.

Titration of natural and immune hemagglutinins

Blood samples were collected from chicks by cardiac puncture. The blood was allowed to clot at room temperature. After spinning down the clot, the serum was pipetted into small tubes and stored in a freezer until time of assay. Serial two-fold dilutions of the serum samples were prepared in 98-well, "U" type, Microtiter plates (Cooke Engineering, Alexandria, Va.). After mixing the diluted serum with an equal amount of a 1% mouse erythrocyte (Mrbc) suspension as antigen, the hemagglutination titers were read.

Cy-treated chicks were bled, at 3, 4, 5, 6, 7, and 10 weeks of age, to test for the presence of natural agglutinins for Mrbc. Blood samples of non-Cy-treated chicks of similar ages served as controls.

The immune hemagglutinin-forming capacity of Cy-treated chicks was tested at 1, 2, 3, and 4 weeks of age by injecting washed Mrbc intracardially and assaying for antibodies in the blood 6 days later. Untreated chickens immunized at the same ages served as controls.

Antigen-sensitive unit (ASU) assay

Other Cy-treated chickens were immunized on the 9th and 11th day of age. Four days later, blood, obtained by cardiac puncture and mixed with an equal volume of Alsever's solution, was assayed for ASU content by the *in vivo* culture method previously described by Seto (15). Donor blood was mixed with antigen (Mrbc) and this mixture was injected intravenously into 12-day embryo hosts. The embryos were bled 6 days later, from the chorioallantoic vein, and the serum was tested for antibody. Blood from non-Cy-treated chickens, immunized 4 days earlier when they were 10 days of age, was assayed similarly and served as controls.

RESULTS

Suppression of natural hemagglutinin formation

The natural hemagglutinin levels of blood during a 3- to 10-week period in three groups of chickens, exposed to 2, 4, and 8 mg of Cy as day-old chicks, and in a control group are shown in Figure 1. A.

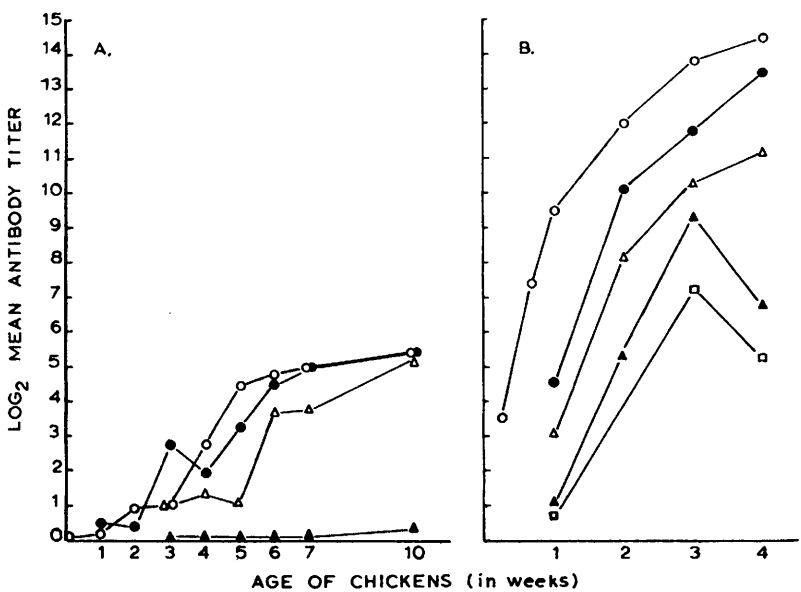

Figure Legend

FIGURE 1. A. Natural anti-mouse hemagglutinin titers of 3- to 10-week control chickens (○) and chickens injected intraperitoneally with 2 mg (●), 4 mg (△), and 8 mg (▲) of cyclophosphamide (Cy) as day-old chicks.

B. Immune anti-mouse hemagglutinin titers of 1- to 4-week control chickens (○) and chickens injected intravenously with 2 mg (●), 3 mg (△), 4 mg (▲) and 5 mg (□) of Cy as 17- to 18-day embryos.

Each group included at least 5 birds. The natural antibody levels of all Cy-treated groups were less than those of the control group during the 4- to 6-week interval. A dose-dependent relationship was evident. By the 10th week, the natural antibody level was negligible in the 8 mg Cy-treated group, whereas near normal levels were observed in groups treated with smaller doses of Cy.

Suppression of immune hemagglutinin formation

The hemagglutinin responses, to immunization with Mrbc, of 1- to 4-week-old normal chickens and those treated with graded doses of Cy as embryos are compared in Figure 1. B. It is apparent that, in the age groups tested, immune responsiveness was less in Cy-treated birds than in untreated birds, and that the decrease was roughly related to the dose. At the higher Cy doses, the onset of the hemagglutinin-forming potential was delayed and recovery was incomplete. Of the Cy-treated chickens immunized at 4 weeks of age, 1 of 6 in the 3 mg Cy-treated group, and 7 of 11 in the 4 to 5 mg Cy-treated groups responded weakly. When these birds were sacrificed and dissected, the bursae of the weakly responding birds were found to be considerably reduced in weight, *i.e.*, less than 0.5 g as compared to the normal mean weight of 1.3 g in control birds of the same age.

TABLE 1. *Hemagglutinin titers of immunocompetent cells of blood from immunized Cy-treated and untreated chicken donors cultured 6 days in embryo hosts.*

	Cy-treatment of donors (mg)[a]			
	0	2	3	4
Donors				
Number tested	6	4	4	4
Age immunized (in days)[b]	10	11	9	9
Embryo hosts				
Number tested	31	38	23	23
Number with antibody	28	19	4	2
Mean antibody titer[c]	6.1	3.5	0.7	0.1

[a] Donors injected with Cy at 17- to 18-days of embryonic age.

[b] Age of donor chicks at time of antigen injection.

[c] Antibody titer in $\log_2$ units.

Suppression of ASU production

The relative ASU content was estimated from the antibody titers of embryo hosts. It was demonstrated earlier that antibody titers obtained in the *in vivo* system are directly related to the amount of immunocompetent units (ASU) transferred (15). Results of the assay for ASU in blood of Cy-treated donors and non-Cy-treated donors immunized with Mrbc are summarized in Table 1. It is clear from the results that the ASU production was diminished in the Cy-treated birds and that the extent of the reduction depended upon the dose of Cy.

DISCUSSION

Varying degrees of immunologic deficiency were observed during the first few weeks post-hatching among chickens treated with cyclophosphamide (Cy) as embryos and day-old chicks. The natural agglutinin levels were considerably less than normal, the onset of the hemagglutinin-forming potential that normally occurs soon after hatching (13) was delayed a week, and the growth of the immune capacity, as measured by hemagglutinin titers and ASU production of immunized birds, was suppressed. With respect to the three parameters of immune potential investigated, the magnitude and duration of the immunosuppression were clearly dependent on the dose. With low Cy doses the immunologic depression was modest and recovery to near normal levels occurred in a few weeks. The recovery pattern, characterized by a suppression of several days duration and followed by an exponential growth response, has been observed in bone marrow, lymphopoietic tissues, and other rapidly proliferating systems exposed to high concentrations of Cy (16, 17). With massive Cy doses suppression was exaggerated and recovery was incomplete; this observation confirms the findings of Lerner and Weidanz (9). Bursae of Fabricius were considerably reduced in birds with obviously depressed immunity.

The nature of the immunosuppressive actions of Cy is incompletely understood. Like X-irradiation, it is a nonspecific destructive agent, and proliferating systems are especially susceptible to its poisoning action (20). Bone marrow aplasia, epithelial destruction, and lymphoid tissue depletions are some of the major sequelae of exposure to high concentrations of this

alkylating agent. Various tissues of the embryonic, neonatal, and adult animals are nonselectively damaged. It has been reported that the GVH capacity is less susceptible to suppression by Cy (9), but our own experiments (14), in accord with those of others (16-19), indicate that tissue immunity, the GVH reactivity, and hematopoietic colony forming efficiency are effectively suppressed by this drug. The suppression of the GVH capacity by Cy in moderate doses is not lasting and recovery to near normal levels occurs in several weeks (14).

A study of this preliminary type unfortunately provides limited insight into the nature of the Cy-produced damage which results in immunologic deficiencies. During the embryonic and early post-hatching period, hemopoietic progenitor cells differentiate into several cell types necessary for immunologic competence (20, 21). It appears that antibody formation in the immunocompetent individual is dependent on the synergistic interaction of two or three cell types, *viz.*, the antigen-processing macrophage, the thymus-derived antigen reactive cell, and the antibody precursor of bone marrow origin (22, 23). Maturation of the antibody-forming tissues in chickens is intimately dependent on the action of the bursa of Fabricius (10, 24, 25) during the perinatal period. The immunologic deficiencies observed in this study are more likely associated with the susceptibiliy of the hemopoietic progenitor cells and multipotential stem cell precursors than with the destruction of immunocompetent cells, which are relatively infrequent in embryos and newly hatched chicks. The immunosuppression produced by Cy in older animals may be due, in part, to the inactivation of cells at this level, as well as to direct damage of immunocompetent cells. Cy-induced immunosuppression was accompanied by bursal hypoplasia which could very well have contributed to the poor development of the immune potential. It is known that suppressive action of cytotoxic agents and the poor recovery that follows their use are intensified in the absence of the bursa (10, 11, 26).

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