APPARENT RESISTANCE OF CHICKEN HEMAGGLUTININ RESPONSE TO IMMUNOSUPPRESSANTS METHOTREXATE AND AZATHIOPRINE

C. D. Barrett and F. Seto

Department of Zoology, University of Oklahoma, Norman, Oklahoma

Cyclophosphamide (CY), methotrexate (MTX), and azathioprine (AZ) were compared as to effectiveness in suppressing the primary hemagglutinin-forming capacity of baby chicks. High doses were lethal to embryos and baby chicks; MTX and AZ were more toxic to embryos than to baby chicks. Week-old chicks, exposed as embryos to CY, were immunologically deficient. CY administered a day after antigenic stimulation to three-day chicks and to immunocytes in *in vivo* culture depressed antibody responses. Conversely, high doses of MTX, AZ, and 6-mercaptopurine, in single and multiple injections, did not suppress this response, and in two instances it was mildly enhanced. The primary hemagglutinin-forming potential of developing chicks was suppressible by CY, but not by MTX or AZ.

Cyclophosphamide (CY), methotrexate (MTX), and azathioprine (AZ) are commonly used in clinical and experimental immunosuppression. CY, a transport form of nitrogen mustard, is an effective immunosuppressant whether administered before or after antigenic stimulation (1,2). MTX, a powerful folic acid antagonist, blocks the interconversion of folate and its derivatives by inhibiting the enzyme tetrahydrofolic dehydrogenase (3). AZ, a purine analog, upon conversion *in vivo* to 6-mercaptopurine (6MP), interferes with purine metabolism (4). The antimetabolites, MTX and AZ, are effective in immunosuppression only if administered after antigenic stimulation (5,6) and the effectiveness varies with the dose, the injection schedule, the antigen, and the experimental animal (2,7). Cells of the lymphoreticular system and other rapidly proliferating tissues actively involved in DNA, RNA, and protein synthesis are especially susceptible to these cytotoxic agents (2,8).

Although chemical immunosuppression has been investigated extensively in adult and young mammals (2,8,9), little is known of the effectiveness of these agents in immature animals in general and in birds in particular. It has been reported that CY treatment of embryos and baby chicks suppresses the development of immunity (10, 11) and that MTX and AZ modify the graft-versus-host reaction in 6-week chickens (12). This paper reports the relative effectiveness of CY, MTX, and AZ in suppressing the hemagglutinin response of developing chicks.

MATERIALS AND METHODS

Baby chicks and young adult birds were hatched from fertile White Leghorn eggs purchased from a local hatchery. Eggs were incubated in an electric incubator and chicks were housed in a heated battery brooder and, later, in small cages.

Immunization of embryos and baby chicks

Mouse erythrocytes (Mrbc) from the albino Swiss strain served as the antigen. Mrbc were obtained by cardiac puncture, with blood drawn into an equal volume of sterile Alsever's solution. The cells were washed in 0.15 *M* NaCl three times and a 1% suspension was prepared for injection. One-half milliliter (0.5 ml) of the Mrbc suspension was administered intracardially into one- and two-week-old chicks and, similarly, 1 ml was injected into three- and four-week-old birds. Serum samples were collected six days after immunization and stored in a freezer for at least two days prior to antibody titrations.

Administration of drugs

Cyclophosphamide (CY), methotrexate (MTX), azathioprine (AZ), and 6-mercaptopurine (6MP) were obtained through the courtesy of the Cancer Chemotherapy National Service Center. All drug solutions were prepared in Hank's balanced salt solution. Except for CY, which dissolved readily, 1 *N* NaOH was required to solubilize the drugs; the excess alkalinity was neutralized with 1 *N* HCl to attain a final pH of 8.0 to 8.5. Solutions were injected intraven-

ously into chick embryos, through windows cut in the shell, and intracardially into baby chicks. The dose and number of injections varied in different experiments.

Determination of antibody response of drug-treated embryos

Various amounts of MTX and AZ were administered to 17-day embryos. Embryos of the same age treated with CY served as positive immunosuppressant controls; others injected with Hank's salt solution served as sham-treated negative controls. A week after hatching, the surviving chicks were immunized with Mrbc, and six days after receiving the antigen they were bled for serum samples. Hemagglutination titers were determined as described for assay of the immunocyte response below.

Determination of antibody response of drug-treated chicks

In the initial experiment to test the effects of MTX, AZ, and 6MP, week-old chicks were immunized. One day later, some received a single injection of the drug and others were given daily injections of the drug on three successive days. To test the effect of the drugs on even younger birds, three-day chicks were immunized, and after a one-day interval were treated with CY, AZ, MTX or 6MP on two successive days. A group of untreated birds served as controls. Serum was collected six days after immunization. Hemagglutination titers were determined as described below for the assay of immunocyte response.

Assay of immunocyte response

The antibody response of immunocytes which had been transferred from immune birds to embryo hosts was determined by the previously described *in vivo* method (13). Two-month-old allogeneic donors were immunized with two injections of 1 ml of a 5% Mrbc suspension given a week apart. A week after the second injection, whole blood, in which antigen-reactive cells should be abundant (14), was obtained by cardiac puncture in Alsever's solution. The blood was centrifuged; the supernatant material was discarded; a sterile 1% suspension of Mrbc in Alsever's solution was added to restore the original blood volume.

The donor-antigen mixture was inoculated intravenously into the chorioallantoic vessel of 14-day embryos. On the following day the drug was administered, and six days later the serum was harvested. For antibody determination, each serum sample was titrated, in duplicate, by the direct hemagglutination test, against a 1% Mrbc suspension in U-type Microtiter plates (Cooke Engineering, Alexandria, Va.). The student t test was used to determine statistically significant differences between the means of experimental and control groups.

RESULTS

Drug-associated mortality

The mortality data for chicks injected with various doses of drugs as 17-day embryos or as week-old chicks are summarized in Table 1. The table presents, first, the number of treated embryos, the number hatched, and the number of these chicks surviving to age 13 days. The second part of the table gives the number of baby chicks which survived for six days after drug treatment. When compared by milligram of drug per gram of body weight (mg/g equivalents), it is apparent that the drugs differed in their toxicity for embryos and chicks. MTX was most toxic for embryos, with LD_{50} at ten days post-treatment being about 0.0009 mg/g; AZ was less toxic, with LD_{50} about 0.09 mg/g; CY was least toxic, with LD_{50} about 0.25 mg/g. All of the seven chicks treated with 0.2000 mg/g CY, the single dose tested, survived. Of the 34 chicks receiving 6MP, in a range of 0.0450 to 0.1800 mg/g, 30 survived.

Effect of drug treatment of embryos on antibody response

Table 2 shows the results of the experiment designed to ascertain if the antimetabolite treatment of embryos can delay development of the immune function of baby chicks, as observed earlier with CY treatment (10,11). In the experiment, maximum dosage was limited by drug toxicity, as indicated by the LD_{50} figures given above. Good immunosuppression was observed in chicks which had been exposed to CY when they were 17-day embryos, but no immunosuppression was apparent after MTX or AZ treatments. There was some

indication of enhancement of the immune response by AZ.

Effect of drug treatment of baby chicks on antibody response

As shown in Table 3, in week-old chicks no immunosuppression was obtained with the drugs despite high dosage and repeated injections. With the two highest doses of AZ there was enhancement of antibody production in these chicks.

In 3-day chicks CY treatment resulted in significant immunosuppression, but there was no evidence of suppression by either AZ or 6MP. Only one bird of the 12 injected survived the MTX treatment and, in this one case, the immune response was depressed.

Effect of drug treatment on immunocytes

Results of the *in vivo* assay of the antibody response of immunocytes are shown in Table 4. As in other experiments in which embryos and baby chicks were exposed to the drugs, there was significant depression of the response after treatment with CY but not with either AZ or MTX.

DISCUSSION

The toxicity of the drugs for embryos and baby chicks varied. Cyclophosphamide (CY) was the least toxic. It has been reported that 14-day embryos, 18-day embryos, and day-old chicks can tolerate 2 mg, 4 mg, and 8 mg of CY, respectively, but that increasing mortality occurs with

TABLE 1. *Survival of embryos and baby chicks after drug treatment.*

	Drug treatment	Dosage (mg/g)[a]	Embryos treated	Chicks hatched[b]	Survivors/total
Embryos	None	0	5	5	4/5[c]
	CY	0.2300	12	6	6/12
		0.1150	9	9	9/9
	MTX	0.0039 - 0.0300	22	17	0/22
		0.0020	13	10	3/13
		0.0009	31	26	16/31
		0.0004	31	27	21/31
		0.0002	23	19	18/23
	AZ	0.4600 - 0.9200	30	1	1/30
		0.1764 - 0.2300	13	5	5/13
		0.0882	12	11	7/12
		0.0014 - 0.0440	35	26	23/35
Chicks	None	0	—	—	28/31[d]
	CY	0.2000	—	—	7/7
	MTX	0.0300	—	—	8/13
		0.0075	—	—	7/8
		0.0009 - 0.0018	—	—	20/22
	AZ	0.0450 - 0.1800	—	—	71/72
	6MP	0.0450 - 0.1800	—	—	30/34

[a]Milligrams of drug per gram of body weight.
[b]Number of treated embryos that hatched.
[c]Number of chicks surviving 13 days after hatching/number of embryos injected.
[d]Number of chicks surviving 6 days after treatment/number of week-old chicks treated.

TABLE 2. *Immune hemagglutinin production of week-old chicks which as 17-day embryos were treated with drugs.*

Drug treatment	Dosage (mg/g)[a]	Number of chicks	Mean titer of serum[b]
None[c]	0	31	8.1 ± 1.29
CY	0.2300	6	3.5 ± 1.90[d]***
	0.1150	9	3.3 ± 2.59[d]**
MTX	0.0009	10	9.2 ± 1.24[d]*
	0.0004	6	9.4 ± 1.16[d]*
	0.0002	5	8.8 ± 0.84
AZ	0.0882	7	7.4 ± 1.44
	0.0220	6	8.5 ± 1.73
	0.0055	7	8.0 ± 1.15
	0.0014	4	8.3 ± 0.96

[a]Milligrams of drug per gram of body weight.
[b]Antibody titer in $\log_2$ units with standard deviation.
[c]Controls injected with Hank's salt solution.
[d]Levels of significance (P); *, 0.05; **, 0.01; ***, 0.001.

higher doses (11,13,15). The estimated LD_{50} for CY in embryos and chicks was about 0.25 mg/g. Methotrexate (MTX) and azathioprine (AZ) were highly toxic to chick embryos with LD_{50} of 0.0009 mg/g and 0.09 mg/g body weight, respectively. In contrast, baby chicks were more tolerant to both drugs and survived single and multiple injections of doses several times greater than that lethal to embryos. Three-day chicks were intermediate in resistance to the toxic effects compared to week-old birds. This increased resistance of week-old birds is undoubtedly related to the rapid emergence, soon after hatching, of functional systems that are involved in detoxification and body defense.

CY readily suppressed the development of the hemagglutinin response in baby chicks, when they were treated as embryos many days before antigen stimulation, in baby chicks treated after hatching, and in immunocytes in the *in vivo* transfer system (10,11). This drug has been shown to be effective also in suppressing the graft-versus-host reaction in 6-week chicks (12) and in embryos (15).

The antimetabolites, MTX, AZ and 6MP, on the other hand, failed to suppress the hemagglutinin response under comparable testing conditions. This was unexpected since MTX and AZ are effective in immunosuppression (8,9,12). Several suggestions are offered to explain the apparent failure of MTX and AZ to suppress the hemagglutinin response of young chicks. (a) Excessive toxicity of the drug to the chicken system may not have permitted the use of amounts adequate to be immunosuppressive. This is the case in some animals (17). (b) The plasmacytoid system may be less suppressible by antimetabolites than the lymphocytoid system whereas both systems are readily suppressed by CY (2, 11,12,15,16). The proliferation of pyroninophilic cells during the humoral immune

TABLE 3. *Immune hemagglutinin production of 3-day- and week-old chicks treated with drugs a day after immunization.*

Age of chicks	Drug treatment	Dosage (mg/g)[a]	Number of injections	Number of chicks	Mean titer of serum[b]
3 days	None	0	—	7	7.9 ± 1.59
	CY	0.2000	1	8	2.2 ± 1.19[c***]
	AZ	0.1680	2	9	7.1 ± 1.73
	6MP	0.1100	2	6	7.8 ± 1.40
	MTX	0.0250	2	1	5.5
1 week	None	0	—	31	8.1 ± 1.29
	AZ	0.1800	3	10	10.6 ± 1.11[c**]
		0.0900	3	18	9.8 ± 1.72[c*]
		0.0450	3	10	9.1 ± 1.33
		0.1800	1	10	9.0 ± 1.91
		0.0900	1	8	8.6 ± 1.98
		0.0300	1	6	8.1 ± 2.20
	6PM	0.1800	3	10	8.6 ± 0.91
		0.0900	3	6	9.1 ± 1.20
		0.0450	3	8	8.6 ± 0.32
	MTX	0.0300	3	8	8.7 ± 1.46
		0.0075	3	8	9.3 ± 2.93
		0.0018	3	5	7.3 ± 3.19
		0.0018	1	9	7.2 ± 1.40
		0.0009	1	6	8.1 ± 1.40

[a]Milligrams of drug per gram of body weight.
[b]Antibody titer in $\log_2$ units with standard deviation.
[c]Levels of significance (P); *, 0.05; **, 0.01; ***, 0.001.

TABLE 4. *Antibody titers of serum of drug-treated 14-day embryos serving as* in vivo *cultures of antigen-reactive immunocytes.*

Drug treatment	Dosage (mg/g)[a]	Number of embryos	Mean titer of serum[b]
None	0	5	11.0 ± 0.80
CY	0.0600	5	8.5 ± 1.32[c]
AZ	0.0220	6	10.8 ± 0.87
MTX	0.0002	5	10.8 ± 0.76

[a]Milligrams of drug per gram of body weight.
[b]Antibody titer in $\log_2$ units with standard deviation.
[c]Level of significance (P), 0.05.

response is inhibited by CY but not by MTX (18). (c) The formation of 19S antibody may be less susceptible to the action of antimetabolites. Treatment with MTX tends to inhibit 7S antibody formation and to prolong 19S antibody production (19,20). The primary hemagglutinin response of baby chicks involves predominately 19S antibody (21,22). (d) Some antimetabolites are less effective as immunosuppressants in rabbits, in guinea pigs and under certain conditions in dogs and mice as well (17,23,24,25,26). The resistance in these cases is possibly due to rapid enzyme inactivation of drugs (27). Chickens may show a similar species difference.

Enhancement of hemagglutinin response was observed in two experiments with MTX and AZ. The enhancement in baby chicks exposed to AZ after immunization was not unexpected in view of reports of others (7,12,16) but its occurrence in chicks treated as embryos many days before antigen stimulation was surprising. A comprehensive discussion of the factors and possible mechanisms in the induction of immune enhancement is available in the report of Makinodan and co-workers (2).

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