THE EFFECT OF AMINOMETHYLPTEROYLGLUTAMIC ACID ON THE DEVELOPMENT OF SKIN HYPERSENSITIVITY AND ON ANTIBODY FORMATION IN GUINEA PIGS

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The inhibition of antibody formation by antimetabolites has been reported in some species. Schwartz *et al.* demonstrated the suppression of antibody response to bovine serum albumin in rabbits employing 6-mercaptopurine (1); 8-azaguanine has been found to decrease hemolysin and precipitin production in mice (2). *In vitro* antibody synthesis has been blocked by amino acid analogues (3), 8-azaguanine (4), or 5-bromouracil deoxyriboside (5).

That aminomethylpteroylglutamic acid (methotrexate) might inhibit immunologic responses has been suggested by several lines of investigation. Folic acid deficiency has been found to impair antibody production in rats (6, 7) and chicks (8). Haas *et al.*, using methotrexate in mice, noted modification of normal LCM[1] virus-host and tumor-host relationships (9–12). Other work in mice indicates that methotrexate decreases mortality due to homograft reactions following lethal total body x-irradiation and homologous marrow inoculation (13).

Recent publications have reported the development of a form of delayed skin reactivity following injection of small amounts of protein antigens in Freund's adjuvant (14), and that this reaction is diminished, but not completely suppressed, in guinea pigs by 200 r total body x-irradiation (15). Development of the delayed sensitivity is followed by production of antibody, a response which is suppressed by 200 r total body x-irradiation (15, 16). A definite relationship between the development of delayed sensitivity and the initiation of production of antibody has not been established.

The present paper describes the suppression in guinea pigs of both the delayed response and antibody production by administration of methotrexate.

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[1] LCM, lymphocytic choriomeningitis.

Methods and Materials

Animals.—Guinea pigs were 200 to 300 gm male Hartley albinos.

Reagents.—The NIH standard diptheria toxoid was employed for sensitization. For skin testing, Massachusetts Department of Health purified diphtheria toxoid, lot KP59a (50 Lf/cc, 1730 Lf/mg N) obtained through the courtesy of Dr. James A. McComb, was used. Ovalbumin, 5X recrystallized, was obtained from Nutritional Biochemical Corporation, Cleveland. A lyophilized heat-killed preparation of BCG tuberculoprotein and rabbit antihuman gamma globulin were provided by Dr. H. Baer of the Division of Biologics Standards, National Institutes of Health. Human gamma globulin was obtained, courtesy of the American Red Cross, and 4-amino-N^{10}-methylpteroylglutamic acid was purchased as methotrexate from Lederle Laboratories Division of American Cyanamid, Pearl River, New York.

Sensitization.—Guinea pigs were injected in their foot-pads with a total of 1 Lf of toxoid or 3 μg ovalbumin in 0.4 cc of equal volumes of buffered saline and either Freund's incomplete adjuvant (8.5 parts of bayol F, 1.5 parts arlacel A) or Freund's complete adjuvant (incomplete + 4 mg BCG tuberculoprotein per animal).

Skin Testing.—Animals were injected intradermally with 0.1 ml containing 25 μg of ovalbumin or 5 Lf of diphtheria toxoid. Tests were read at 6 and 24 hours and recorded as the diameters of erythema and induration in millimeters. Reactions greater than 10 mm in 2 diameters were consider positive.

Determination of Presence of Antibody.—For eliciting systemic anaphylaxis an intravenous injection of 0.25 cc of a 4 mg/ml solution of ovalbumin was employed. Active cutaneous anaphylaxis (ACA) (17) was performed by intradermal injection of 25 μg of either diphtheria toxoid or ovalbumin following injection of 0.25 ml of 2 per cent Evan's blue intravenously. 20 minutes after inoculation of the antigen, animals were sacrificed and skin sites examined for relative accumulation of dye as compared to control sites injected with 25 μg of the heterologous antigen. Dye accumulations greater than 10 mm in diameter were considered positive. Guinea pigs were tested for antibody to diphtheria toxin by a toxin neutralization test. Animals were injected with dilutions of a standard toxin and skin sites read at 42 hours. In all cases, only one test was performed per animal and animals were either skin-tested or examined for antibody.

Recording of Temperature.—Rectal temperatures of animals, inoculated with antigen in complete adjuvant as previously indicated and, after 7 days, injected intraperitoneally with 4 mg of antigen, were determined as described by Uhr (18).

Histologic and Blood Studies.—White blood cell (WBC) counts were determined in a Levy hemocytometer. Wright's stained smears were examined for differential counts. Hemoglobin was determined in a Sahli hemoglobinometer. Skin sections were stained with hematoxylin and eosin and examined with the assistance of Dr. Ruth Kirschstein of the Division of Biologics Standards, National Institutes of Health.

Methotrexate Dose Schedules.—Animals were injected intraperitoneally every 48 hours with 1.7 mg or 5 mg of methotrexate in 1 cc of Ringer's solution. Controls received 1 cc of Ringer's solution by the same route. Treatment was started 2 days before inoculation of antigen.

RESULTS

Systemic Effect of Methotrexate in Guinea Pigs.—All guinea pigs in the experiments described survived 14 days administration of the 1.7 mg or 5 mg dose schedules of methotrexate. Animals lost about 10 per cent of their original body weight during the first 4 days of administration of the drug, but by 1

TABLE I

Effect of Methotrexate on Skin Reactions of Guinea Pigs Injected with 1 Lf of Diphtheria Toxoid in Incomplete Adjuvant

Size (mm) of skin reaction at 6 and 24 hours following skin test.*

Day after immunization	Not treated		Methotrexate-treated	
	6 hrs.	24 hrs.	6 hrs.	24 hrs.
5	0	25 × 20	0‡	0
	0	19 × 15	0	0
	0	18 × 14	0	0
	0	17 × 14	0	0
	0	16 × 11		
	0	14 × 12		
6	0	30 × 28	0	0
	0	30 × 28	0	0
	0	25 × 20	0	0
	0	25 × 18	0	0
	0	22 × 20	0	0
	0	20 × 17	0	0
	0	20 × 15	0	0
	0	20 × 12		
	0	20 × 12		
7	0	19 × 18	0	0
	0	19 × 17	0	0
	0	17 × 15	0	0
	0	17 × 10	0	0
	0	15 × 10		
	0	0		
9	25 × 20	0	0	0
	20 × 15	0	0	0
	19 × 12	0	0	0
12	40 × 30	0	0	0
	35 × 30	0	0	0
	30 × 30	0	25 × 23	0
14	35 × 20	0	0	0
	30 × 20	0	0	0
	25 × 15	0	0	0

* All animals skin-tested with 5 Lf toxoid in 0.1 cc.

‡ Reactions of less than 10 mm are reported as 0, as animals inoculated with heterologous antigen had reactions up to this size when tested with toxoid.

week they were back to their original weights; thereafter, there was a weight gain. During the period of methotrexate administration WBC counts and hemoglobins were stable while differential WBC counts changed from 66 per cent lymphocytes before drug administration to 90 per cent after 2 weeks; polymorphonuclear counts dropped from 34 to 10 per cent during this period.

TABLE II

Effect of Methotrexate on Skin Reactions of Guinea Pigs Injected with 3 μg of Ovalbumin in Incomplete Adjuvant

Size of skin reaction at 6 and 24 hours following skin test*

Day after immunization	Not treated		Methotrexate-treated	
	6 hrs.	24 hrs.	6 hrs.	24 hrs.
5	0	22 × 20	0	0
	0	20 × 20	0	0
	0	16 × 15	0	0
	0	10 × 10	0	0
			0	0
			0	0
6	0	30 × 20	0	0
	0	25 × 20	0	0
	0	25 × 20	0	0
	0	22 × 20	0	0
	0	20 × 18	0	0
			0	0
7	0	25 × 20	0	0
	0	25 × 19	0	0
	0	20 × 16	0	0
	0	20 × 15	0	0
	0	0	0	0
			0	0

* All animals skin-tested with 25 μg ovalbumin.

Paper electrophoretic studies of serum protein levels of experimental animals failed to demonstrate any consistent changes during the course of the experiments. These results are in general agreement with a report that guinea pigs are relatively insensitive to the toxic effects of methotrexate (19).

Effect of Methotrexate on Skin Reactions Employing Antigens in Incomplete Adjuvant.—When animals were inoculated with diphtheria toxoid in incomplete Freund's adjuvant and skin-tested up to 2 weeks following injection of the homologous antigen, delayed reactions were noted on days 5 to 7 following inoculation (Table I). After 9 days, Arthus reactions (6 hours) were seen. In

contrast, only 1 of the methotrexate-treated animals which was inoculated with toxoid in adjuvant developed a skin reaction to toxoid greater in size than controls inoculated with a heterologous antigen (ovalbumin).

In order to confirm these results, a test system employing another antigen, ovalbumin, was used. Table II shows results seen in this system when animals were tested on days 5 to 7. The animals receiving methotrexate did not develop delayed reactions during this period.

Effect of Methotrexate on Antibody Production Using Antigens in Incomplete Adjuvant.—14 days after inoculation with ovalbumin or diphtheria toxoid in incomplete adjuvant, animals were tested for antibody by various means. To test for antibody to diphtheria toxin, two methods were employed: either ACA which has been reported by Uhr to be positive in cases where passive cutaneous anaphylaxis and the toxin neutralization test in rabbits have been negative

TABLE III

Effect of Methotrexate on Antibody Formation in Guinea Pigs Injected with 1 Lf of Diphtheria Toxoid in Incomplete Adjuvant

Test for antibody	Not treated	Methotrexate-treated
Active cutaneous anaphylaxis (ACA)	3/3*	0/6
Toxin neutralization	8/9	0/15

* Reported as: Number positive for antibody over total tested. All animals tested on the 14th day following immunization.

(15); or toxin neutralization in the skin of the guinea pig being tested for antibody. This test will detect as little as 3.5×10^{-5} units of antitoxin when the minimum dose of standardized toxin which gives a positive reaction is used (20). Results of these tests for antibody are reported in Table III. Diphtheria antitoxin was not detected by either test in animals receiving methotrexate whereas it was found in all but 1 of the control animals.

In order to test for antibody to ovalbumin, systemic anaphylaxis and ACA were employed. In addition, controls to indicate whether methotrexate itself could inhibit anaphylaxis were included. Animals were, therefore, injected with ovalbumin in incomplete adjuvant and given 1.7 mg of methotrexate 2 days before being tested; also, animals inoculated with ovalbumin in adjuvant and given a complete 2 week, 1.7 mg course of methotrexate, were passively sensitized with rabbit antihuman gamma globulin serum 12 days after primary injection and 2 days later challenged with human gamma globulin. Results are shown in Table IV. Again, none of the animals receiving methotrexate were positive in tests for antibody. Among controls, all animals were found to have antibody. Systemic anaphylaxis occurred in passively sensitized animals which had received a single 1.7 mg dose of the drug.

Effect of Methotrexate on Skin Reactions and on Antibody Production Employing Toxoid in Complete Adjuvant.—Unlike the results seen when incomplete adjuvant was employed, animals inoculated with toxoid in complete adjuvant developed delayed hypersensitive reactions to toxoid despite treatment with the 1.7 mg regimen of methotrexate (Table V). In most of the animals, however, this dose of methotrexate was sufficient to block the antibody response as determined by ACA tests. These findings resemble those in studies of irradiated animals in which antibody production, but not delayed hypersensitivity, was blocked (15, 16).

TABLE IV

Effect of Methotrexate on Antibody Formation in Guinea Pigs Injected with 3 μg of Ovalbumin in Incomplete Adjuvant

Treatment	Test on 14th day after immunization	Results	
		Fraction showing fatal anaphylaxis*	Fraction showing ACA‡
None	i.v., ovalbumin or ACA	3/3	3/3
Methotrexate§	i.v., ovalbumin or ACA	0/6	0/6
Methotrexate on 12th day only	i.v., ovalbumin	3/3	—
Methotrexate + rabbit anti-human gamma globulin on day 12§	i.v., human gamma globulin	3/3	—

* Reported as fatal reaction/total tested.

‡ Reported as positive ACA/total tested.

§ 1.7 mg methotrexate every 48 hours commencing 2 days before immunization.

In order to determine whether delayed skin reactivity following injection of antigen in complete Freund's adjuvant could be blocked by methotrexate, an experiment was performed using a dose schedule of 5 mg of the drug every 48 hours. Table VI shows the results of skin tests with toxoid in these animals. The larger dose of methotrexate was sufficient to inhibit the development of a delayed skin reaction and of antibody production following inoculation with toxoid in complete adjuvant. Histologic studies of skin test sites in this experiment showed that sections from animals given methotrexate were indistinguishable microscopically from untreated controls tested with a heterologous antigen (ovalbumin), while controls tested with homologous antigen (toxoid) had marked mononuclear infiltrates.

Specific Febrile Response.—Uhr has shown that guinea pigs develop a specific febrile response when they are inoculated intraperitoneally with a large amount of an antigen to which they have a delayed hypersensitive response (18). In order to determine whether methotrexate would block this response a group

TABLE V

Effect of 1.7 Mg Methotrexate Regimen on Skin Reactions and Antibody Response in Guinea Pigs Injected with 1 Lf of Diphtheria Toxoid in Complete Adjuvant

Day after immunization	Test	Size of skin reaction at 24 hrs.*	
		Not treated	Methotrexate-treated
		mm	*mm*
7	Skin test, diphtheria toxoid, 5 Lf	35 × 23	32 × 28
		30 × 25	22 × 20
		27 × 25	20 × 15
		25 × 25	20 × 12
		25 × 20	18 × 17
		22 × 17	17 × 14
		22 × 17	15 × 12
		20 × 18	13 × 13
		20 × 17	0
		15 × 11	0
		Results of ACA	
14	ACA, for diphtheria antitoxin	5/6‡	1/6

* Animal skin-tested with 5 Lf of toxoid in 0.1 cc.

‡ Reported as number positive for ACA over total tested.

TABLE VI

Effect of 5 Mg Methotrexate Regimen on Skin Reactions and Antibody Response in Guinea Pigs Sensitized with 1 Lf of Diphtheria Toxoid in Complete Adjuvant

Test	Day after immunization	Size of skin reaction			
		Not treated		Methotrexate-treated	
		6 hrs.	24 hrs.	6 hrs.	24 hrs.
		mm	*mm*	*mm*	*mm*
Skin test, toxoid 5 Lf	7	0	22 × 20	0	0*
		0	22 × 18	0	0
		0	20 × 17	0	0
		0	20 × 15	0	0
		0	20 × 12	0	0
	11	0	17 × 10	0	0
		0	15 × 13	0	0
		0	0	0	0
	15	35 × 20	0	0	0
		30 × 20	0	0	0
		25 × 19	0	0	0
		Results of ACA test			
ACA for antitoxin	16	6/7‡		0/3‡	

* Reactions of less than 10 mm are reported as 0, as animals inoculated with heterologous antigen had reactions up to this size when tested with toxoid.

‡ Reported as number positive for ACA over total tested.

of 5 animals was inoculated with ovalbumin in complete Freund's adjuvant, treated with the 5 mg regimen of methotrexate, and 7 days after inoculation, when delayed reactivity is strong, given a 4 mg intraperitoneal injection of ovalbumin. The results of this experiment are illustrated in Fig. 1. A group of 6 untreated control animals specifically challenged with ovalbumin responded with a temperature rise as reported by Uhr (18). Animals given methotrexate and challenged with homologous antigen (ovalbumin) had small temperature rises similar to the pattern of 10 control animals challenged with 4 mg of heterologous antigen—5 human gamma globulin–sensitized animals were tested with

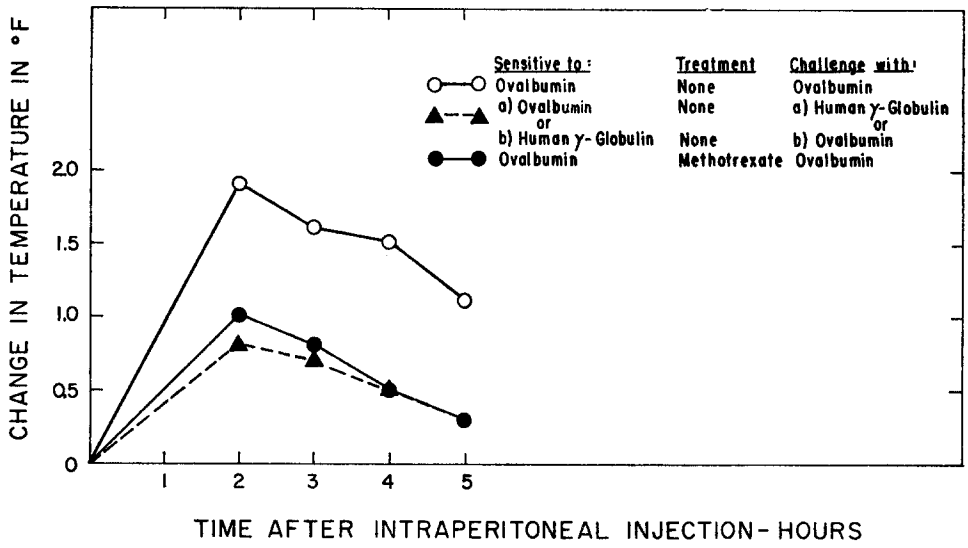

FIG. 1. Specific febrile response in methotrexate-treated guinea pigs. 6 ovalbumin-sensitive positive controls were challenged intraperitoneally with 4 mg of ovalbumin. 5 animals were inoculated with ovalbumin, treated with the 5 mg regimen of methotrexate, and challenged with 4 mg of ovalbumin and 10 negative controls, sensitized with either ovalbumin or human γ -globulin, were challenged with the heterologous antigen. The average temperature elevations of the three groups are shown. Test was performed 7 days after primary inoculation.

4 mg of ovalbumin, and 5 ovalbumin-sensitized animals, with human gamma globulin.

Similar results have been found employing toxoid.

DISCUSSION

The effects of methotrexate on the immunologic responses of guinea pigs apparently depend upon the strength of the antigenic stimulus and upon the dose of methotrexate employed. Suppression of the delayed response following injection of antigen in complete adjuvant requires more of the drug than is

needed to suppress the delayed response following inoculation with antigen in incomplete adjuvant. The antibody response is more easily inhibited in guinea pigs by methotrexate than is the delayed hypersensitive response. In this respect the effect of methotrexate resembles that of x-irradiation(15, 16).

It is interesting to note that methotrexate does not seem to inhibit immunologic reactions in rabbits (21), a finding which may be analogous to the situation which exists with 8-azaguanine which inhibits antibody production in mice, but not in rats (2). These differences in drug sensitivity may relate to species differences in susceptibility of enzymes to methotrexate, to the rate of detoxication of methotrexate, or to any of several other possible explanations (19).

The exact mechanism by which methotrexate suppresses immunologic responses in guinea pigs is obscure, as folic acid derivatives are important in many biochemical processes (22), and methotrexate acts as a folic acid inhibitor (19). Since, however, the synthesis of a new protein (here, antibody) involves formation of nucleic acid, it is of significance that methotrexate inhibits nucleic acid synthesis. Schwartz postulated that inhibition of nucleic acid synthesis may be the basis for the action of 6-mercaptopurine to depress antibody formation in rabbits (1). The fact that delayed sensitivity is not completely suppressed in guinea pigs by an LD_{50} of x-irradiation (15) may be due to the relative radioresistance of the biochemical processes for nucleic acid synthesis (23).

Suppression of the development of the delayed hypersensitive state might be a useful tool in the investigation of such biological phenomena as the mechanism for antibody synthesis (24), autoimmunity, the rejection of homografts (25), and the host reaction to infection (26). The observation that methotrexate inhibits the specific febrile response in addition to inhibiting the delayed skin reaction, may indicate that methotrexate acts to suppress development of the delayed sensitive state and not simply delayed skin reactivity.

SUMMARY

1. In guinea pigs, aminomethylpteroylglutamic acid (methotrexate) is capable of blocking the development of delayed skin hypersensitivity, the primary antibody response, and the specific febrile response to ovalbumin and diphtheria toxoid. The primary antibody response is more easily inhibited than is the development of delayed skin hypersensitivity.

2. The effect of methotrexate on immunologic responses depended upon the dose of methotrexate employed and the strength of the antigenic stimulus.

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