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STUDIES ON IMMUNOSUPPRESSION BY PURINE NUCLEOSIDE ANALOGUES—II

EFFECTS ON SKIN-GRAFT REJECTION AND IMMEDIATE HYPERSENSITIVITY IN MICE*

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Abstract—The effects of several purine nucleoside analogues on skin-graft rejection and immediate hypersensitivity in mice have been investigated. It was found, by measuring tritiated thymidine uptake into the DNA of lymphoid tissues, that β-D-arabinofuranosyl-6-mercaptopurine (Ara-6-MP) and the periodate oxidation product of β-D-ribosyl-6-methylthiopurine (MMPR-OP) induced only partial impairment of the cell proliferation taking place in the lymph nodes draining a skin graft. Suppression by β-D-ribosyl-6-methylthiopurine (MMPR) was much more effective. Complete suppression of rapidly dividing precursors of cytotoxic lymphocytes was found. The biochemical blockades imposed by these compounds are somewhat limited and recovery from inhibition was relatively rapid, sometimes leading to enhanced proliferative activity. The blockade imposed by Ara-6-MP obviously can be easily overcome; extension of drug treatment had no effect on the duration of the blockade. MMPR-OP seems to have a protective effect on the graft itself since pretreatment of the skin-graft donor increases graft survival on the recipient significantly compared to treatment of the recipient only. A good deal of the pharmacological action of Ara-6-MP seems to be on nonspecific peripheral mechanisms, as indicated by its anti-inflammatory activity in the Arthus type of hypersensitivity and its protective effect against general anaphylaxis. Anti-complementary activity was excluded as a possible explanation for this effect of Ara-6-MP.

DEPENDING on the experimental design and the species investigated, the action of antimetabolites on various manifestations of the immune response may be different.

Purine nucleoside analogs like Ara-6-MP§ and MMPR-OP suppress the immune response to skin grafts under conditions where the humoral antibody response of mice stimulated with tanned sheep red blood cells is not inhibited.[1,2] Under certain experimental conditions similar discrepancies in the susceptibility of delayed hypersensitivity vs. humoral antibody formation have been reported for 6-MP in guinea pigs and rabbits.[3,4] Although the immune response to skin grafts is not completely understood, it seems to be initiated by competent, small lymphocytes

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§Abbreviations used: Ara-6-MP, β-D-arabinofuranosyl-6-mercaptouurine; MMPR, β-D-ribosyl-6-methylthiopurine; MMPR-OP, the periodate oxidation product of MMPR; 6-MP, 6-mercaptopurine; TCA, trichloroacetic acid; BSA, bovine serum albumin; SRBC, sheep red blood cells; ^{3}HT, tritiated thymidine.

from the recirculating pool. After stimulation by transplantation antigens, the lymphocytes home in the draining lymph node, transform into actively dividing large immunoblasts, and finally reach the end stage of the immunologically committed effector cell.[5,6] The measurement of tritiated thymidine incorporation into DNA has been used in the present study to evaluate the interaction of purine nucleoside analogs with the proliferative events that have been shown to take place, primarily in the draining lymph nodes, in response to a skin graft.[7–9] Distant lymph nodes and spleen also seem to be involved, but to a minor degree.[7,8,10] Pharmacological manipulation of delayed hypersensitivity may also be on nonspecific, peripheral mechanisms of recognition and destruction of the grafted tissue. The present study was undertaken in order to evaluate the relative effects of our drugs on these two parameters, namely, cellular proliferation in lymphoid tissues and peripheral events such as inflammatory reactions.

MATERIALS AND METHODS

Animals. The mice used in this study were purchased from Jackson Laboratories, Bar Harbor, Me. Skin-graft experiments were performed with female AKR mice. Only animals of at least 20 g and not more than 30 g were used. AKD_2F_1 (AKR/J ♀ x DBA/2J ♂) females were used as skin-graft donors. The animals were vaccinated against mouse-pox virus on arrival and streptomycin was added to their drinking water for a period of 5 days.

Drugs. Drugs were prepared as described previously.[11] Drug levels and schedules were based on previous studies concerned with toxicity and drug metabolism.[1,2,12–14] Sublethal and/or near toxic levels were chosen.

Skin grafting. AKR mice were orthotopically grafted on their tails with 2 × 15-mm skin patches from AKD_2F_1 donors, as described by Bailey and Usama.[15] Problems concerning the vascularization of the grafts are minimized with this simple technique. This system represents a histocompatibility barrier in the direction $AKD_2F_1 \rightarrow$ AKR ($H\text{-}2^d$ $H\text{-}2^k$ alleles on the donor AKD_2F_1 mice vs. $H\text{-}2^k$ $H\text{-}2^k$ alleles in the recipient AKR mice). The different schedules of drug treatment are described with the results. Graft-survival time in days was scored as positive until complete breakdown took place.

Graft vs. host reaction. The graft vs. host assay described by Simonsen[16] was used. Spleen cells ($2{\cdot}6 \times 10^7$) from adult mice were injected i.p. (in a volume of 0·1 ml) into 8-day-old LAF_1 (C57L/J ♀ × A/HeJ ♂) mice. Each litter of mice constituted a separate experiment. They were injected with either syngeneic LAF_1 spleen cells, untreated C57L spleen cells, or C57L cells treated with various doses of Ara-6-MP or MMPR-OP. Spleen cells from donor animals were teased out into Krebs–Ringer buffer at 0°. To 0·2 ml of a suspension of about $3{\cdot}25 \times 10^7$ cells, 0·1 ml of glucose (19·8 mg/ml) and 0·7 ml drug solution were added. The final concentration of Ara-6-MP was 40 μg/ml ($1{\cdot}41 \times 10^{-4}$M), 80 μg per ml ($2{\cdot}82 \times 10^{-4}$M) or 160 μg/ml ($5{\cdot}64 \times 10^{-4}$M). The final concentration of MMPR-OP was 80 μg/ml ($2{\cdot}55 \times 10^{-4}$M). After incubation for 20 min at 37°, the spleen cells were washed twice with 3 ml of Krebs–Ringer buffer to remove free drug. Cell viability, as determined by trypan blue exclusion, was not significantly altered by this treatment. Injected animals were killed 8 days later and relative spleen indices were determined by dividing the spleen index (in mg

per g body weight) of each animal by the mean spleen index of the negative control group injected with LAF_1 cells.

Tritiated thymidine incorporation. Tritiated thymidine (Schwarz Bioresearch, Inc., Orangeburg, N.Y.) was given at 1 μc/g body weight (226 μc/μmole) 1 hr before sacrifice. Three experimental groups were tested: (1) sham-grafted mice (isologous transplants) (2) nondrug treated allografted mice, and (3) allografted mice treated with MMPR, Ara-6-MP, or MMPR-OP, with various drug schedules and doses. At the designated times, the mice were killed by cervical dislocation; the tissues were weighed and their DNA was extracted by a technique modified from that of Kimball *et al.*[17] as follows: The tissues were homogenized in ice cold 5% TCA and centrifuged for 2 min at 1470 *g*. The precipitate, after being washed four times with 5 vol. of cold 5% TCA, was washed twice with 2 ml of 95% ethanol and dried partially *in vacuo* in order to remove the alcohol. It was then suspended in 0·8 ml of 10% sodium chloride, and 0·1 ml of 0·1 M sodium hydroxide was added to make the solution 0·02 M in NaOH. Heating for 1 hr in a boiling water bath hydrolyzed RNA and extracted DNA. The suspension was centrifuged for 4 min at 1470 *g*. The DNA in the supernatant was precipitated with 3 vol. of cold 95% ethanol. The DNA precipitate was centrifuged 20 min at 1800 *g* in the cold, washed with 1·5 ml of 95% ethanol and dissolved in 0·5 ml of 0·1 M NaOH. DNA was reprecipitated by adding 1 drop of 6 N hydrochloric acid. Centrifugation was performed in the cold for 20 min at 1800 *g*, and the pelle of purified DNA was finally redissolved in 0·5 ml of 0·1 M sodium hydroxide. The quantities of reagents given are adequate for the small amounts of tissue (approximately 10 mg wet weight) obtained from lymph nodes. When the spleen and thymus were extracted, the volumes of the reagents had to be proportionally increased, and after extraction and hydrolysis at 100° only fractions of the whole extract were further processed. The end result was then calculated back to the original amount of tissue. To the above solution of purified DNA, 10 ml of a scintillation-counting solution consisting of 2, 5-diphenyloxazole, 1, 4-*bis*-2-(4-methyl-5-phenyloxazolyl) benzene, toluene and ethyl alcohol was added. Radioactivity was measured in a liquid scintillation counter (Nuclear-Chicago). Each sample was counted for 3 1-min periods and checked within 10%. Samples of tissues to be extracted were weighed separately and pooled for DNA preparation. The lymph nodes designated as draining lymph nodes include the 2 lumbar nodes and the caudal node (nomenclature of Dunn[18]). As samples of distant lymph nodes, the brachial and inguinal lymph node pairs were chosen.

Even though the variation of results from animal to animal within an experimental group was considerable, sufficient animals were tested and sufficient points were obtained to establish clear-cut trends in the proliferative activity of the tissues tested.

Skin tests. Control and drug-treated mice received 3 i.p. injections of 5 mg of bovine serum albumin (Sigma Chemical Company) with a 4-day interval between each (days 0, 4 and 8). On day 33, their flanks were shaved with electric clippers and a razor. The next day, 1 mg of BSA in 0·05 ml of 0·9% saline was injected intradermally with a 27-gauge needle.[19] Mice with a positive Arthus reaction were bled by heart puncture and their serum was checked for precipitating antibodies by interfacial ring-tests and pooled. This pool contained 0·35 mg of antibody nitrogen per ml as determined by quantitative precipitation.[20] A scoring system described by Benedict and Tips[19] was used. Positive Arthus reactions were scored when the skin lesions appeared within 4 hr.

In an attempt to evoke a local passive Arthus reaction, up to 0·07 mg of antibody nitrogen, followed within 20 min by the same quantity of BSA-nitrogen, was injected intradermally at the same site. Positive skin reactions repeatedly failed to occur in control mice.

Measurements of complement inhibition. Barbital buffered saline, pH 7·4, containing optimal concentrations of Ca^{++} and Mg^{++} at 37° was used for complement (C′) fixation. Titrations were carried out by using the 50 per cent hemolysis ($C'H_{50}$) assay. Anti-C′ activity was measured by the method described by Yachnin.[21]

RESULTS

Skin graft studies. The results are summarized in Table 1. Treatment of both the

TABLE 1. EFFECT OF MMPR, MMPR-OP and ARA-6-MP ON AKD_2F_1 SKIN ALLOGRAFT SURVIVAL IN AKR MICE*

Experiment†	Daily treatment	Days of drug administration	Toxic deaths	Mean graft survival (days) ± average deviation	T/C (%)	P
I	Saline control	− 5 to 0	0/10	15·4 ± 1·0		
	MMPR-OP, 65 mg/kg	− 5 to 0	1/10	16·4 ± 1·3	106	N.S.
II	Saline control	− 4 to + 1	0/8	16·1 ± 1·4		
	MMPR-OP, 40 mg/kg	− 4 to + 1	0/8	22·7 ± 2·6	141	<0·05
	MMPR-OP, 40 mg/kg	− 7, − 4, − 1, +2	0/8	19·6 ± 1·4	105	N.S.
	MMPR-OP, 40 mg/kg	− 4, − 1, + 2, + 5	1/8	23·1 ± 2·3	144	<0·05
	MMPR-OP, 40 mg/kg	− 2, + 1, + 4, + 7	0/8	22·1 ± 3·3	137	<0·05
III	Saline control	− 2, + 1, + 4, + 7	0/6	16·2 ± 0·6		
	MMPR-OP, 40 mg/kg	− 2, + 1, + 4, + 7	0/8	23·6 ± 1·9	146	<0·05
IV	Saline control	− 4, − 1, + 2, + 5	0/10	14·4 ± 2·2		
	MMPR-OP, 65 mg/kg	− 4, − 1, + 2, + 5	1/10	20·9 ± 3·5	145	>0·05
	MMPR-OP, 65 mg/kg	− 3, 0, + 3, + 6	0/10	17·9 ± 1·8	116	<0·05
V[2]	Saline control	− 1 to + 3	0/8	14·6 ± 1·0		
	MMPR-OP, 75 mg/kg	− 1 to + 3	0/7	23·6 ± 1·9	162	<0·01
VI[2]	Saline control	− 1 to + 4	0/7	13·1 ± 0·9		
	MMPR-OP, 60 mg/kg in 2 divided doses daily	− 1 to + 4	0/7	16·3 ± 1·6	125	>0·05
	MMPR-OP, 100 mg/kg	− 1 to + 2	3/6	19·7 ± 1·7	150	<0·05
VII	Saline control	− 4, − 1, + 2, + 5	0/10	14·4 ± 2·2		
	MMPR-OP, 65 mg/kg; 65 mg/kg given to skin donors days − 5 to − 1	− 4, − 1, + 2, + 5	1/10	28·3 ± 5·3	197	<0·001
VIII[1]	Saline control	0 to slough	0/7	14·4 ± 1·4		
	Ara-6-MP, 60 mg/kg	0 to slough	0/7	19·0 ± 1·1	136	<0·05
IX	Saline control	0 to slough	0/10	14·4 ± 2·2		
	Ara-6-MP, 60 mg/kg	0 to slough	0/10	17·9 ± 3·6	124	<0·05
	Ara-6-MP, 60 mg/kg; 150 mg/kg given to skin donors days − 5 to − 1	0 to slough	0/10	18·1 ± 4·5	126	<0·05
X[2]	Saline control	− 1 to + 4	0/7	13·1 ± 0·9		
	MMPR, 30 mg/kg	− 1 to + 4	0/6	17·0 ± 1·6	130	<0·05
XI[1]	Saline control	0 to + 5	0/7	14·0 ± 1·4		
	MMPR, 25 mg/kg	0 to + 5	0/7	21·0 ± 0·4	150	<0·001

*Skin grafts were performed on day 0. MMPR and MMPR-OP were given in single daily doses unless otherwise noted. The daily dose of Ara-6-MP was divided and given 8 hr apart. Toxic deaths, No. of toxic deaths/No. of mice in group. T/C, graft survival in drug-treated mice/graft survival in nontreated mice. P values were calculated by the method of Hogben.[22] N.S., not significant.

†The superscript numbers indicate the references from which data have been taken to facilitate comparisons.

skin donor and recipient with MMPR-OP (Exp. VII) resulted in a significant increase in prolongation of skin-graft survival over treatment of recipients alone. This might be due to the capacity of MMPR-OP to bind to tissues.[14] Ara-6-MP also shows binding capacity,[23] but pretreatment of the donor did not affect the host's response (Exp. IX). Different doses of MMPR-OP (40 mg/kg and 65 mg/kg) given on similar drug schedules did not result in differences in graft survival (Exp. II and IV). Timing of the treatment seems to be much more important. With pretreatment only, there is practically no effect (Exp. I). As soon as part of the postoperative period was covered by the drug schedule, better results were obtained (Exp. II). Administration of relatively high doses of MMPR-OP during days − 1 to + 3 was quite effective (Exp. V). When MMPR-OP was given with a 3 day interval between individual doses, certain regimens gave better results than others. (Exps. II–IV). Previous skin graft experiments with MMPR were included for comparison (Exps. X and XI).

Incorporation of tritiated thymidine into the DNA of lymphatic tissues during the response to skin grafts. At various times during the response to a skin allograft and during recovery from drug treatment, the proliferative events in draining lymph nodes, distant lymph nodes, spleen, and thymus were followed by measuring the 1-hr uptake of tritiated thymidine. The results of these experiments are summarized in Figs. 1 to 4. A clear-cut response was obtained in draining lymph nodes, which showed much higher uptake of tritiated thymidine than did sham-grafted control mice (Fig. 1A).

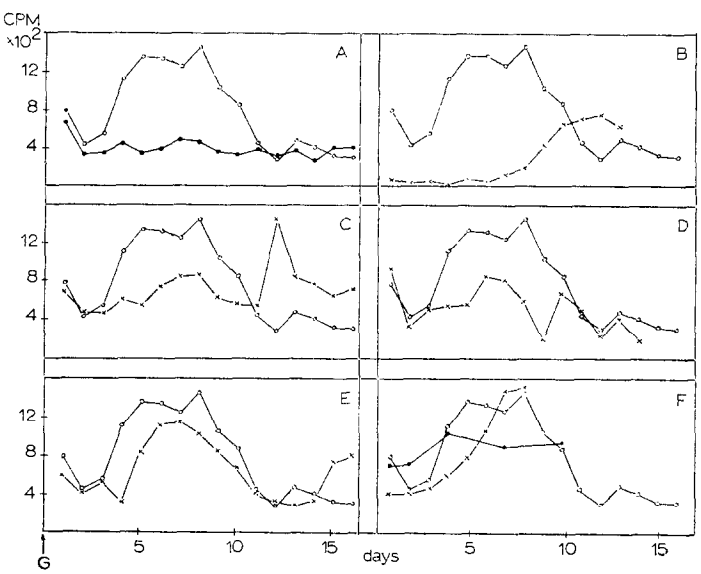

FIG. 1. Incorporation of ^{3}HT into DNA of draining lymph node tissue after 1 hr labeling. ○———○, untreated homograft control; ●———●, A: Sham-grafted (isografted control), F: Ara-6-MP, 250 mg/kg/day from day − 1 to slough in 2 divided doses daily; ×———×, B: MMPR, 35 mg/kg/day from day − 1 to + 4, C: MMPR-OP, 65 mg/kg/day from day − 5 to − 1, D: MMPR-OP, 65 mg/kg/day at day − 4, − 1, + 2 and + 5, E: Ara-6-MP, 120 mg/kg/day from day − 1 to + 5 in 2 divided doses daily, F: Ara-6-MP, 120 mg/kg/day from day −1 to slough. B–F are allografted and drug-treated. The radioactivity is expressed as total counts per organ. Each value is the mean of two series of experiments, with four animals at each point.

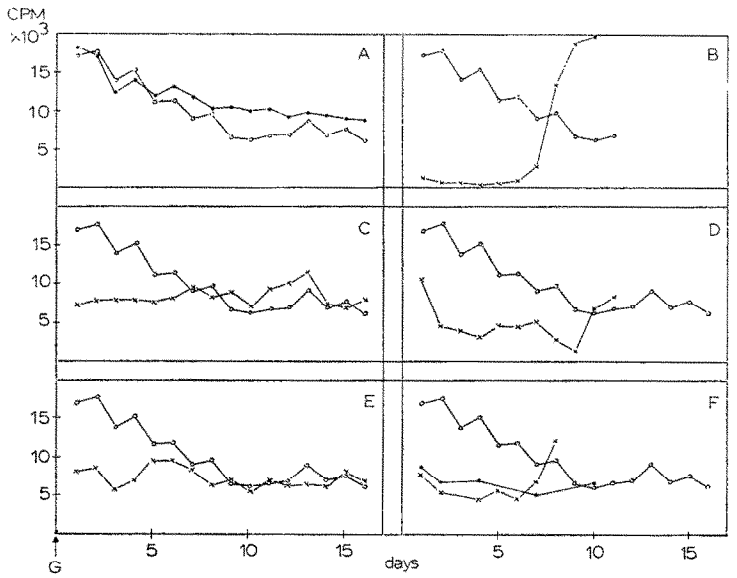

FIG. 2. Incorporation of ^{3}HT into the DNA of distant lymph node tissue. Other information as described in the legend to Fig. 1.

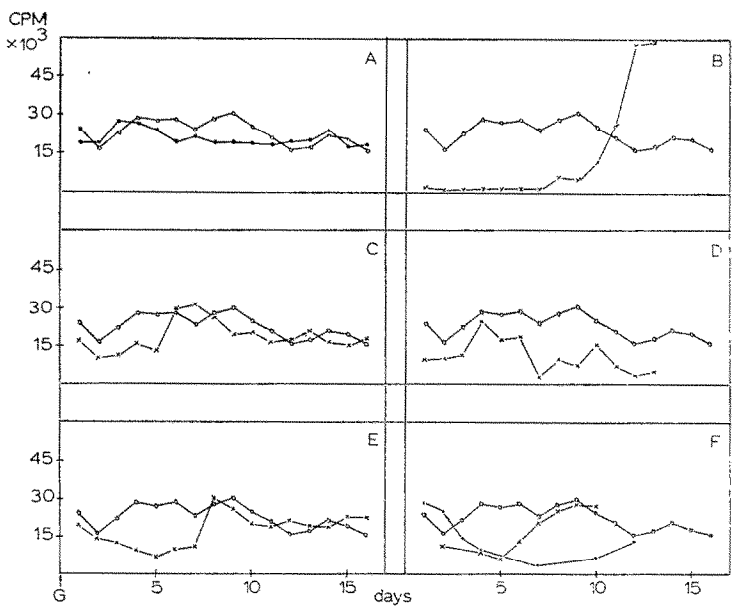

FIG. 3. Incorporation of ^{3}HT into the DNA of spleen tissue. Other information as described in the legend to Fig. 1.

This parallels morphological observations of proliferative processes induced in draining lymph nodes by skin grafts.[7] Increased uptake of tritiated thymidine starts 48 hr after grafting and extends over a period of 8–10 days. As can be seen in Figs. 2A, 3A, and 4A, this effect was small in spleen tissue and was not obtained with distant lymph nodes and thymus.

Treatment with MMPR (35 mg/kg/day) from day − 1 to + 4 (day 0 being the day of grafting) caused signs of toxicity as expressed by an average weight loss of 36 per cent 5 days after treatment was stopped. Moreover, the volumes of all the tissues tested and their proliferative activity was much more effectively depressed with MMPR

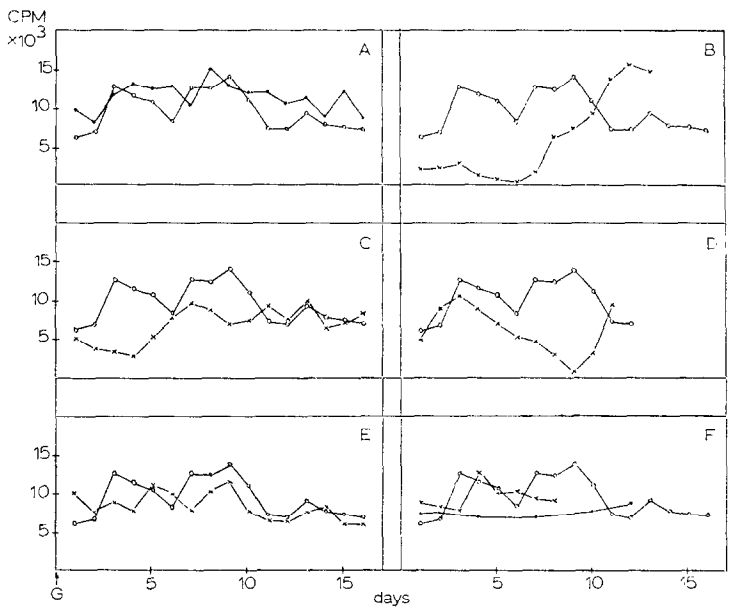

FIG. 4. Incorporation of ^{3}HT into the DNA of thymus tissue. Other information as described in the legend to Fig. 1.

than with any of the other drugs studied (Figs. 1B to 4B). Recovery took place within 4–7 days after drug administration had been discontinued. Tritiated thymidine incorporation increased quickly and rose to much higher levels than that in nontreated control mice. This particular recovery pattern was found with distant lymph nodes, spleen, thymus, and to a lesser extent with draining lymph nodes.

In an analogous experiment, MMPR-OP was tested using two different treatment schedules. One was with pretreatment only (65 mg/kg/day given from day − 5 to − 1). Toxicity with this regimen was negligible, consisting only of a delayed and transient 12 per cent weight loss in the treated mice. In contrast, administration of the same drug dose on days − 4, −1, + 2, and + 5 led to progressive weight losses, which reached 35% at the end of the experiment. The pretreatment schedule resulted in a partial, relatively long-lasting impairment of DNA syntheses in draining and non-draining lymph nodes and in the thymus (Figs. 1C, 2C, and 4C). However, comparatively little depression was found in the spleen (Fig. 3C). Termination of the MMPR-OP

inhibition of DNA synthesis in draining lymph nodes was immediately followed by an explosive increase of proliferation (Fig. 1C). Treatment at 3-day intervals, extending into the postoperative period, generally produced more efficient inhibition (Figs. 1D to 4D) and there was no enhanced proliferation in the draining lymph nodes after recovery. These findings parallel the results from skin-graft survival studies (Table 1, Exps. I and IV).

Treatment with Ara-6-MP, from day − 1 to day + 5, caused only partial inhibition of DNA synthesis at a level of 120 mg/kg/day, given in two divided daily doses. (Figs. 1E to 4E.) The spleen and distant lymph nodes were as sensitive to Ara-6-MP as the draining lymph nodes. Enhanced proliferations after the drug activity had disappeared was observed in draining lymph nodes, but not in the other tissues. Treatment with Ara-6-MP at 120 mg/kg/day until slough of the grafts did not prolong the impairment of DNA synthesis (Figs. 1F to 4F), indicating increased drug resistance in the population of immunocompetent cells from day 7 onward. Treatment with higher doses of Ara-6-MP (250 mg/kg/day till slough) extended the period of impaired DNA synthesis in all the tissues studied, but drug resistance was again observed by day 11. Toxicity to mice, as expressed by weight loss (up to 10 per cent) at this dose level, was brief.

Graft vs. host reaction. Ara-6-MP has been shown to bind to cell surfaces and to induce non-specific hemagglutination.[23] The possibility of a direct action by Ara-6-MP on cytotoxic lymphocyte-target cell interactions was considered and this hypothesis was supported by the observation that Ara-6-MP slowed the elimination of homologous C3H spleen cells in AKR mice.[24] Furthermore, MMPR-OP was found to bind to cell surfaces to a higher degree than Ara-6-MP.[14] Parental spleen cells were incubated *in vitro* in solutions of Ara-6-MP and MMPR-OP prior to injection into 8-day-old F_1 hybrid mice, as described in *Materials and Methods.* From Table 2, it is evident that coating of donor lymphoid cells with drugs does not prevent the graft vs. host reaction in the recipient mice.

Effect of MMPR, MMPR-OP and Ara-6-MP on immediate hypersensitivity and complement activity. These experiments were undertaken in order to test for possible anti-inflammatory activities. Groups of 36 mice were hyperimmunized with 3 i.p.

TABLE 2. EFFECT OF PREINCUBATION WITH DRUGS ON THE ABILITY OF PARENTAL SPLEEN CELLS TO PRODUCE SPLENOMEGALY IN F_1 HYBRID MICE*

Pretreatment of donor spleen cells	%of control splenomegaly
40 μg/ml Ara-6-MP	104
80 μg/ml Ara-6-MP	94
160 μg/ml Ara-6-MP	106
80 μg/ml MMPR-OP	90

*Eight-day-old litters of LAF_1 mice were divided into negative control ($LAF_1 \rightarrow LAF_1$), positive control ($C57L \rightarrow LAF_1$), and drug-treated groups. They were injected with the appropriate donor spleen-cell suspensions, which had been previously incubated 20 min with or without drug at 37° and washed twice with buffer. The spleen indices of the positive controls varied between 3 and 4. Each value is the average of results from 2 litters.

injections of bovine serum albumin in complete Freud's adjuvant, on days 0, 4, and 8. Drug treatment was comparable to that used in skin-graft experiments: MMPR (35 mg/kg/day) and Ara-6-MP (135 mg/kg/day in two divided doses) were injected daily starting 6 days before skin testing, which took place at day 34. MMPR-OP was given daily at 65 mg/kg/day, starting 4 days before skin testing. The results are presented in Table 3. In response to the challenging, intradermal BSA injection at day 34, 39 per cent of the sensitized, saline-treated animals died of systemic anaphylaxis Treatment with MMPR and MMPR-OP afforded no protection, but treatment with Ara-6-MP afforded complete protection from death. Anaphylaxis under similar circumstances was obtained when sensitized animals were injected intralabially.[25] The surviving mice were tested for active Arthus reactions and then bled to determine circulatory, precipitating anti-BSA antibody by the interfacial ring-test. Positive Arthus reactions were demonstrated in all of the MMPR-OP-treated mice, in 88 per cent of the MMPR-treated mice, and in 95 per cent of the control mice, which correlated with the percentage of mice showing positive tests for anti-BSA antibody in their sera. Only 6 per cent of the Ara-6-MP treated animals had positive Arthus reactions in spite of positive tests for circulating anti-BSA antibody for the whole group. On the whole, these data suggest effective inhibition of nonspecific inflammation by Ara-6-MP as compared to MMPR and MMPR-OP. In view of the important role of complement in the inflammatory events in Arthus reactions and general anaphylaxis, the interaction of these drugs with C′ was studied.

TABLE 3. ARTHUS REACTION AND ANAPHYLACTIC SHOCK IN DRUG-TREATED MICE ACTIVELY SENSITIZED TO BSA

Treatment before skin testing	Anaphylactic shock		Active Arthus reaction* in surviving mice							Ring-test on the serum of each surviving mouse		
	no. dead / no. mice	% positive	4+	3+	2+	+	±	0	% positive	+	−	% positive
Control	14/36	39	8	1	5	7	0	1	95	22	0	100
MMPR, 35 mg/kg/day for 6 days	19/36	53	6	8	0	0	1	2	88	14	3	78
MMPR-OP, 65 mg/kg/day for 4 days	22/36	61	9	2	3	0	0	0	100	14	0	100
Ara-6-MP, 135 mg/kg/day for 6 days in 2 divided doses daily	0/36	0	0	0	0	1	1	34	6	36	0	100

*Scoring system described by Benedict and Tips.[19]

A known amount of C′ sufficient to cause 100 per cent lysis of optimally sensitized SRBC was used. When SRBC were sensitized with mouse anti-SRBC serum (25 times the amount causing 100 per cent hemolysis) and drug and C′ were added during the second step of the experiment, no effect of either Ara-6-MP or MMPR-OP on C′ activity was observed. There was also no interference with the sensitization of SRBC when the drugs were present during the first phase. The drug concentrations

tested ranged from $1{\cdot}6 \times 10^{-5}$ to 2×10^{-3}M. Anti-C′ activity can therefore be excluded as an explanation for inhibition of general anaphylaxis and active Arthus type of hypersensitivity by Ara-6-MP.

DISCUSSION

The present studies allow two main conclusions to be drawn. First, Ara-6-MP and MMPR-OP interact with peripheral aspects of the immune response and these effects may or may not be an important part of the temporary impairment of graft rejection observed in skin-graft survival studies. Second, Ara-6-MP and MMPR-OP induce only partial impairment of the proliferative response in draining lymph nodes, the central manifestation of rejection.[26, 27] The complete suppression of cell proliferation during treatment with MMPR was followed by enhanced proliferative activity. At the present time, no experimental facts can be put forward to explain this over-compensation in cell proliferation after cessation of MMPR treatment. The biochemical effects of these drugs, which are discussed in the preceding paper,[11] do not lead to a total blockade of nucleic acid synthesis, nor do they destroy basic elements of cellular function. Many synthetic pathways continue to function and large pools of precursors may build up. As a result, general imbalance may cause cell death, as suggested by some investigators.[28–30] But if drug treatment is stopped in time, the imbalance might be corrected by acceleration of the blocked pathway. Another possibility; namely, reutilization of nucleic acids released from injured cells, has also been proposed as a cause of enhanced antibody production after drug treatment.[31] This hypothesis is supported by the findings that polynucleotides are directly incorporated into lymphocytes[32] and that nucleic acids or their digests, after incorporation into living bacterial cells, stimulate cell division.[33] Both of these possibilities may apply in the case of MMPR and MMPR-OP. Ara-6-MP, on the other hand, is not cytotoxic at the dose levels used and therefore reutilization of nucleic acids on a large scale seems unlikely to occur with respect to the delayed enhancement effects obtained with Ara-6-MP.

Based on previous toxicity studies,[2, 12] sublethal drug levels and schedules were chosen for the skin-graft studies. Nevertheless, cytotoxicity cannot be excluded as part of the immunosuppressive mechanism. Many of the significant differences between the individual drugs studied may be related to their mode of interaction with cellular metabolism. In general, the results parallel our findings on the time dependence of optimal suppression of antibody synthesis in spleen cells, reported in the preceding paper.[11] The drugs seem to selectively suppress the rapidly dividing precursors of cytotoxic lymphocytes. MMPR has an indirect effect on DNA-synthesis by feedback inhibition of *de novo* purine synthesis.[34, 35] This blockade seems to be quite effective as long as a sufficient drug level is maintained, but obviously does not lead to severe damage to immunocompetent cells. Therefore recovery is rapid. The same is true for Ara-6-MP, which has only one demonstrable blockade—it inhibits cytidine diphosphate reductase by 50–80 per cent.[13] Such a restricted target area can easily be passed by since the drug is relatively rapidly excreted. The observation that extension of Ara-6-MP treatment had no effect on the duration of effects in lymphoid tissues seems to confirm this.

MMPR-OP, which is a dialdehyde, might mimic alkylating agents and cross-link macromolecules involved in cellular proliferation.[14] Such an effect not only could

be responsible for slower recovery from drug inhibition, but also could explain the activity against the proliferative response to allografts, even when the drug was given before grafting. The finding that MMPR-OP was much more effective when both the skin donor and the recipient were treated supports the concept that part of the action of MMPR-OP may be on the peripheral aspects of immunity. Similar results have been found with cortisone[36] and thalidomide.[37] The latter acylates naturally occurring diamines and therefore might bind to proteins.[38] We found that significant amounts of MMPR-OP were still bound to the nucleo-proteins of mouse-tail skin 4 days after a single, i.p. injection of drug.[14] Thus, inhibition of peripheral sensitization could take place at the site of grafting, as suggested by Medawar's model.[39] On the other hand, short exposure *in vitro* of spleen cells to MMPR-OP did not interfere with their capacity to induce a graft vs. host reaction. In the case of cortisone, delay of lymphatic connection between graft and host,[40] limitation of the tissue destructive activity of lysosomal enzymes,[40, 41] suppression of inflammatory reactions,[42, 43] and impairment of phagocytic function[44] have been suggested as rejection-inhibiting factors. MMPR-OP does not interfere with the inflammatory reactions we have studied, but we are not in a position to make any conclusions with respect to the other possibilities.

Ara-6-MP has also been reported to bind to cell surfaces.[23] Nevertheless, treatment of the donor mice before using their tail skin for grafting did not enhance the prolongation of skin-graft survival obtained by treating the recipients only. Also, incubation of spleen cells *in vitro* with Ara-6-MP did not impair the graft vs. host reaction. In contrast to MMPR-OP and MMPR, Ara-6-MP inhibited general anaphylaxis and the direct, Arthus type of hypersensitivity without diminishing the immune status of the animals. A decrease of the cellular component of the inflammatory response, as it was recently demonstrated for 6-MP,[45] does not seem to provide an acceptable hypothesis for the action of Ara-6-MP, in view of its limited anti-proliferative activity. On the other hand, it does not seem to be necessary to reduce the entire mononuclear cell population in order to suppress inflammation.[3]

Since C′ (complement) may play a part in tissue damage,[46, 48] as well as in histamine release from mast cells,[49] a possible anti-C′ effect of Ara-6-MP was investigated. No such activity could be demonstrated and therefore its anti-inflammatory activity cannot be explained at present.

It has been suggested that inflammatory mechanisms quite similar to those found in immediate hypersensitivity may be involved in allograft rejection,[50–52] but the question of whether or not the suppression of inflammation by Ara-6-MP contributes to the observed prolongation of skin-graft survivals has to remain open. On the whole, a number of effects can be ascribed to the compounds studied. However, their relative importance in prolonging the survival of skin grafts is completely unknown.

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