MECHANISMS IN THE SUPPRESSION OF DELAYED HYPERSENSITIVITY IN THE GUINEA PIG BY 6-MERCAPTOPURINE*

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6-mercaptopurine (6-MP)[1] has been shown to suppress the expression of certain experimental and clinical immune responses. These include delayed cutaneous reactivity to antigen (1), allograft rejection (2), manifestations of certain "autoimmune" disorders (3), and some types of humoral antibody response (4). The mechanisms involved in these suppressive effects of 6-MP are still open to question. Evidence has been presented both for specific immunosuppressive (5) and nonspecific anti-inflammatory (6) actions.

This study describes in vivo and in vitro functional studies in guinea pigs receiving 6-MP after presentation with *Mycobacterium tuberculosis*. This model was chosen to study the effect of 6-MP upon developing cellular immunity after preimmunization, the situation most analogous to the conditions of clinical use. Specific attention was paid to the two major cell type populations known to be important in the manifestations of delayed hypersensitivity, the lymphocytes and monocytes-macrophages (7). Sequential comparative measurements in vivo were made of: (*a*) patterns of skin test reactivity to tuberculin, irritants, and certain vasoactive substances; (*b*) patterns in levels of circulating leukocytes; (*c*) transfer studies involving specific subpopulations of cells; (*d*) several quantitative in vitro measurements of lymphocyte and monocyte reactivities, such as: (*i*) lymphocyte blast cell transformation and proliferation in response to tuberculin or phytohemagglutinin; (*ii*) macrophage inhibition factor (MIF) activity production; (*iii*) macrophage function.

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[1] *Abbreviations used in this paper*: CFA, complete Freund's adjuvant; [^{3}H]Tdr, tritiated thymidine; I.I.I., isotope incorporation index; MIF, macrophage inhibition factor; 6-MP, 6-mercaptopurine; NHS, normal human serum; PBS, phosphate-buffered saline; PHA, phytohemagglutin; PPD, purified protein derivative; SRS-A, slow-reacting substance of anaphylaxis.

Materials and Methods

General Protocol.—

Animals: Albino outbred guinea pigs, 600-850 g in weight, were obtained from the Stuttle Guinea Pig Farm Bristol, Pa., and the Harvard Animal Research Center, Cambridge, Mass.

Induction of sensitization: All animals received on day 0 a total of 1.0 ml of complete Freund's adjuvant (CFA), containing 1.0 mg of heat-killed mycobacteria (H37Ra, Difco Laboratories, Detroit, Mich.) by multiple footpad and subcutaneous injections of 0.1 ml each.

6-MP treatment: The animals were divided into two groups. Group A (105 animals) received 6-MP (kindly supplied by Dr. George Hitchings, Burroughs-Wellcome Co., Research Triangle Park, N. C.). 10 daily doses of 35–50 mg/kg were given on days 4–14 by deep intramuscular injection. Group B (136 animals) received equivalent volumes of the phosphate-buffered NaOH diluent instead of the 6-MP on days 4–14.

Skin Tests.—Animals were tested on the day before receiving CFA and at weekly intervals thereafter until termination of the study: (*a*) Tuberculin skin test reactivity was measured by the intradermal injection of 0/1 ml of PPD-S (5 TU, kindly supplied by Dr. Lydia Edwards, Tuberculosis Section, National Communicable Disease Center, U.S. Public Health Service). In selected studies, PPD (supplied by Mr. G. N. Freirichs, Ministry of Agriculture Fisheries and Food, Central Veterinary Labs, Weybridge, Surrey, England) was also used in various concentrations. Skin test reactions were read at 36 h and expressed as the mean of two perpendicular diameters of induration. (*b*) Skin reactivity to irritants was measured by the injection of 0.1 ml of turpentine-olive oil (1/4:vol/vol ratio); readings were made similar to that for the tuberculin skin test. (*c*) Skin reactivity to vasoactive substances was measured by the intradermal injection of (*i*) 0.02 ml of histamine, 1:10,000; (*ii*) compound 48-80 (3.5 mg/ml, kindly supplied by Dr. A. Kligman, Department of Dermatology, University of Pennsylvania); (*iii*) bradykinin (1, 10, and 50 μg, kindly supplied by Dr. J. Spragg, Robert Breck Brigham Hospital, Boston, Mass.); and (*iv*) the slow-reacting substance of anaphylaxis (SRS-A, 1, 5, and 10 U, kindly provided by Dr. R. Orange, Robert Breck Brigham Hospital). Measurements of wheal responses to these substances were made 20 min after injection. (*d*) Responses to possible chemical mediators of delayed hypersensitivity were measured by intradermal injection of 0.1 ml of material containing MIF activity. This lymphokine was produced by the incubation of 30×10^6 lymphocytes (obtained from lymph nodes of guinea pigs sensitized to tuberculoprotein, with 20 μg/ml of Weybridge PPD [8] in HEPES-buffered Roswell Park Memorial Institute Medium-1640 (Grand Island Biological Co., Grand Island, N.Y.). The culture supernatant was concentrated five times, dialyzed, and shown to contain MIF activity by its ability to reversibly inhibit active migration of normal guinea pig peritoneal macrophages from capillary tubes in vitro. When injected intradermally, this supernatant caused a monocyte infiltrate (9) in the skin of normal, unsensitized animals. Induration was measured at 16 h.

Formed Blood Elements.—Sequential measurements were made weekly of hematocrit, total and differential leukocyte counts, and platelets.

Cell Transfer Studies.—In these experiments, $1.0–3.0 \times 10^8$ cells from normal, unsensitized animals were obtained from (*a*) bone marrow, or (*b*) minced lymph nodes. The bone marrow cells were obtained from the femurs, fibia, and humeri by gentle flushing with RPMI-1640, through a 22-gauge needle. The cell suspension was transferred to 10-ml glass test tubes for settling of particulate material and suspended by passage through a 25-gauge needle. Mesenteric, preaortic, axillary, and inguinal lymph nodes were excised, minced, and passed through fine mesh onto a 5-cm column of glass beads (VirTis 16-220, VirTis Co., Inc., Gardiner, N.Y.) (10). After an incubation period of 15 min at 37°C, the nonadherent node cells, consisting of over 97% small lymphocytes, were eluted from the column at a flow rate of 1–2 ml/min. These cells were injected intravenously into three types of recipient animals: (*a*) guinea pigs of

group A who had received CFA on day 0, followed by daily injections of 6-MP on days 4–14, with cell transfer carried out on days 22–24; (*b*) guinea pigs of group B, sensitized with CFA, followed by placebo; (*c*) normal, unsensitized guinea pigs. As a further control in the cell transfer experiments, selected members of each recipient group received Hanks' solution obtained from the cell washings instead of the bone marrow or lymph node cells from the donors.

In a reverse type of cell transfer experiment, guinea pigs injected with CFA on day 0, followed by 6-MP or placebo treatment on days 4–14, received intraperitoneal injections of 40 cm^3 of light mineral oil (Marcol, or Bayol, Exxon Chemical Co., New York) on day 24–28. 3 days later, bone marrow and peritoneal cells were collected from each animal, washed several times with chilled Hanks' solution, and injected intravenously in amounts of $1.0–3.0 \times 10^8$ cells into normal, unsensitized guinea pigs.

All recipients were skin tested with a series of several doses of PPD (Weybridge) and turpentine-oil 36 h before intravenous cell transfer. 18 h after transfer, the PPD and irritant skin tests were repeated.

Lymphocyte Culture.—The proliferation of lymphocytes cultured either with phytohemagglutinin (PHA) or tuberculin was studied as previously described by us in detail (11). Lymphocytes were cultures for 3 days with PHA (PHA-P, Difco Laboratories, final concentration 1.25 μg/ml) and 5 days with old tuberculin (OT—Jensen-Salsbery Laboratories, Kansas City, Mo. final concentration, 0.5 mg/ml) or control cultures, without any PHA or tuberculin. The incorporation of tritiated thymidine ([^{3}H]Tdr, 6.7 Ci/mM, New England Nuclear, Boston, Mass.) was measured by standard scintillation counting techniques and expressed as an isotope incorporation index (I.I.I.):

$$\text{I.I.I.} = \frac{\text{mean counts per minute in vials containing PHA or tuberculin}}{\text{mean counts per minute in vials containing only control medium}}.$$

In selected cultures, lymphocyte proliferation was also assayed by the percentage of cells incorporating isotope, indicated by autoradiographic techniques. The cell button in each such culture was washed twice, resuspended in normal human serum (NHS), and layered on gelatin-coated slides. These smears were dried, incubated with Kodak NTB-2 (Eastman Kodak Co., Rochester, N. Y.) emulsion for 7 days, developed with Kodak developer (Dektal or D-19), and stained with Giemsa solution. Cells were considered labeled if they contained at least three times the number of grains seen in the background. The incidence of mitotic division was also estimated in selected cell cultures using Colcemid, 20 μg/ml (12). The results were expressed as mitotic figures seen per 1,000 lymphoid cells counted.

MIF Activity Measurements.—

(*a*) The direct measurement of inhibition by tuberculin of the in vitro migration of macrophages obtained from animals in group A or B was carried out by the methods described by David (8). On day 21, animals were injected intraperitoneally with Marcol oil (Humble Oil & Refining Co., Houston, Tex.); 72 h later, the peritoneal cavity was lavaged with heparinized (1 U/ml) chilled Hanks' solution. The peritoneal cells obtained were washed twice, drawn into glass capillary tubes, and centrifuged at 200 *g* for 5 min. The glass capillary tubes were fractured at the cell-medium interface and placed in small lucite or Sykes-Moore chambers (Bellco Glass, Inc., Vineland, N.J.). The chambers were filled with minimal essential medium (Grand Island Biological Co.) or RPMI-1640 (Grand Island Biological Co.) containing either 20% NHS*[2] (as described above) or 15% normal guinea pig serum plus 5% fetal calf serum. Various concentrations of PPD were added to the test chambers. At 24 or 36 h, each chamber was visualized microscopically, and slide images were projected and traced on calibrated paper. The migration area was then cut out and weighed; the weight was used to represent the area of migration. The

[2] NHS* = normal human serum previously heated at 56°C for 40 min.

results were expressed as the percentage of inhibition equal to:

$$1.00 - \frac{\text{migration without antigen} - \text{migration with antigen}}{\text{migration without antigen}} \times 100.$$

(*b*) The indirect measurement of migratory inhibition factor production was determined by a modification of the method of Rocklin et al. (13). Lymph node cells obtained from unsensitized animals and from selected animals in groups A and B were tested for their ability to produce a soluble substance capable of reversibly inhibiting the migration of peritoneal exudate cells obtained from normal, unsensitized guinea pigs. Lymph node cell suspensions were transferred in 10-ml volumes to test tubes containing cotton wool (Fisher Scientific Type A, Fisher Scientific Co., Pittsburgh, Pa.) and incubated at 37°C for 15 min in a 5% CO_2, 10% O_2 atmosphere. The nonadherent cell population was removed, washed twice in medium, and suspended in replicate incubation mixtures containing final concentrations of 18×10^6 cells/3 ml and incubated in 5% CO_2, 10% O_2. The supernatants of each incubation mixture were removed in 12 and 24 h, and concentrated to $\frac{1}{5}$ of the original volume by vacuum dialysis in previously boiled membranes (Arthur H. Thomas Co., Philadelphia, Pa.). They were then dialyzed against fresh medium containing either 20% NHS* or a mixture of guinea pig and fetal calf serum. The dialyzed supernatant was then used to fill lucite chambers containing capillary tubes packed with peritoneal exudate cells obtained from normal, unsensitized guinea pigs by oil installation, as described above. The four types of incubation mixtures were compared: (*i*) node cells plus PPD (Weybridge) 20 μg/ml; (*ii*) node cells without PPD; (*iii*) node-free medium plus PPD; (*iv*) cell-free medium alone.

The areas of migration of guinea pig macrophages incubated with the supernatants from *i*, *ii*, *iii*, and *iv* were measured after 24 h, and the percentage of indirect inhibition caused by the production of MIF by lymph node cells was expressed by the formula:

$$1 - \frac{a}{b} \times \frac{d}{c} \times 100.$$

Macrophage Function Studies.—The macrophages were initially defined morphologically according to the description of Cohn (10). These amoeboid, phagocytic cells contained a single ovoid or indented nucleus and abundant cytoplasm. They were strongly adherent to glass by multiple cytoplasmic processes. Over 90% of this cell type could be shown to actively phagocytize latex particles (1.947 μm, Coulter Electronics Inc., Hialeah, Fla.). The cytoplasm contained vacuoles, oil droplets, and a glistening internal structure.

Phagocytosis: 15×10^6 peritoneal cells with the morphologic characteristics of macrophages were suspended in 5.0 ml of RPMI-1640 medium, containing 20% NHS. They were incubated for 3 h in the presence of ^{14}C microcombusted particles (final concentration, 1 μCi/ml, New England Nuclear Corp.), in 15-ml sealed Erlenmeyer flasks under 5% CO_2, 10% O_2 at 37°C. Cells adhering to the glass walls of the chamber were then washed five times with Hanks' solution, removed by freezing and thawing, and transferred to glass fiber/filter discs for drying, radioactivity being subsequently determined by standard scintillation spectrometry methods. The results are expressed as counts per minute of ingested radioactive carbon per 10^6 macrophages. Estimates of the number of cells adherent to the glass surface were made by direct counting with a calibrated eyepiece and determining the protein content of the freeze-thaw effluent.

In additional experiments, macrophages were placed in 1.0 ml of medium in Leighton tubes containing flying cover slips. One drop of standardized latex particles (1.947 μm, Coulter Electronics, Inc.) was added. Incubation of the medium was continued for 2 h at 37°C, under 10% O_2, 5% CO_2. The cover slips were removed, washed twice in phosphate-buffered saline (PBS), fixed in 6% gluteraldehyde, and observed by phase contrast microscopy for phagocyto-

sis of the latex particles. Parallel cultures incubated in the absence of latex particles were observed for morphologic alteration (11).

Adherence: 15×10^6 peritoneal macrophages, defined by morphology and the ability to phagocytose latex particles, were suspended in NHS* and passed on to a 5-cm column of alcohol, KOH-cleaned glass homogenizing beads (VirTis no. 16-220). After incubation under 10% O_2, 5% CO_2 for 45 min, the columns were washed with 4 vol of 0.2% EDTA in buffered saline free of calcium and magnesium (14). The degree of adherence was estimated by the calculation:

$$\frac{\text{number of macrophages added} - \text{number of macrophages nonadherent}}{\text{number of macrophages added}} \times 100.$$

Migration rate: Peritoneal exudate cells were suspended in acid-cleaned glass tubes of precise internal diameter (160 nm, Drummond Scientific Co., Broomall, Pa.), sealed with Critoseal (Arthur H. Thomas), and centrifuged at 200 *g* for 5 min. The tubes were placed in lucite chambers containing RPMI-1640 in 20% NHS*, carefully held in the horizontal position, and observed under microscopy with a calibrated eyepiece for the rate of migration of the advancing front of macrophages within the tube. Movement was expressed in terms of millimeters per 24 h.

Viability: Phase-contrast microscopy criteria (15) and eosin exclusion were used. Evaluation was made of both (*a*) those cells adherent to and spreading on glass and capable of phagocytosing latex particles, and (*b*) nonadherent cells in the fluid phase.

RESULTS

Skin Test Reactivity.—

(*a*) Skin test reactivity to tuberculin was markedly suppressed by 6-MP administration (Fig. 1). Animals in group B had positive skin tests to PPD starting as early as day 14, with almost all animals reactive by day 21. In contrast, animals in group A showed little skin test reactivity to PPD for up to 6 wk after discontinuation of 6-MP therapy.

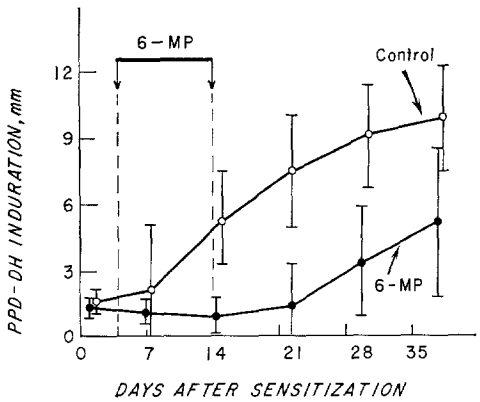

FIG. 1. Effect of 6-MP administration on the development of delayed hypersensitivity to tuberculoprotein. Prolonged suppression of skin test reactivity to 5 TU PPD was found in group A (●) animals, often lasting several weeks after the cessation of 6-MP administration.

(*b*) To test for the nonspecific suppression of skin test reactivity by 6-MP, a variety of additional agents were used (Table I):

Irritant skin tests: Group B animals showed marked erythema and induration 36 h after intradermal injection of turpentine-oil. This response was significantly less ($P < 0.05$) in group A animals, although the differences were not as marked as seen in tuberculin skin test responses. Comparative measurements were made by a disc micrometer of the anterior-posterior diameters of the posterior footpads of groups A and B animals 21 days after CFA had been injected into these sites. The average diameter for the group A animals was approximately 40% of that seen in group B, which suggested a depressed, nonspecific, inflammatory response to the injected CFA.

TABLE I

Effect of 6-MP on Skin Test Reactivity

	Mean diameter of induration on day					
	1	7	14	21	28	35
			mm			
Turpentine:oil	19/18	16/19	13/20	15/21	16/20	20/21
Histamine	16/14	15/17	16/18	17/16	18/17	16/15
48–80	13/13	14/13	15/14	8/7	14/15	16/14
SRS-A	15/12	14/13	13/16	11/14	13/12	ND‡
Bradykinin	9/8	10/8	11/14	10/9	ND	ND
Lymphokine§	10/11	8/12	2/10	4/12	6/14	8/17
PPD	2/2	2/2	2/5	2/7	3/9	5/11

* Group A animals/group B animals (groups of at least six animals).

‡ Not done.

§ Lymphokine produced by lymphocyte interaction with antigen and known to contain macrophage inhibition factor activity.

Vasoactive mediator skin tests: Histamine, compound 48–80, SRS-A, and bradykinin: No differences were noted between group A and group B animals in the wheal responses seen 20 min after intradermal injection of these agents.

The lymphokine skin test: Lymphokine from lymphocyte cultures failed to induce significant responses in the skin of group A animals during the period in which PPD skin test reactivity was markedly suppressed. No suppression in response was seen in group B animals when compared with normal, unsensitized animals. However, with the development of systemic sensitivity to tuberculoprotein, the reaction to lymphokine increased, perhaps reflecting a response to the tuberculin contained in it.

Blood Cellular Elements.—There was a rapid and marked decrease in the level of circulating monocytes in group A animals when compared with the level of those cells in animals of group B (Fig. 2). A similar pattern of suppression was noted in the levels of hemoglobin, granulocytes and platelets. The level of large

lymphocytes (diameter greater than 10 μm) was moderately suppressed in group A animals. The suppression was most apparent on day 14, and was followed by mild rebound elevation by day 28. In contrast, the level of small lymphocytes (6–8 μm diameter with characteristic morphology) was only moderately suppressed in group A (Fig. 3). Conversely, monocytes, granulocytes, platelets, and lymphocytes increased slightly in group B animals.

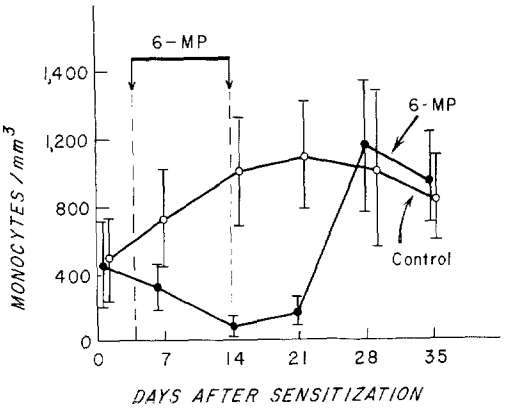

FIG. 2. Effect of 6-MP administration on the circulating monocyte level. A rapid and dramatic suppression of total monocyte count was found in group A (●) animals.

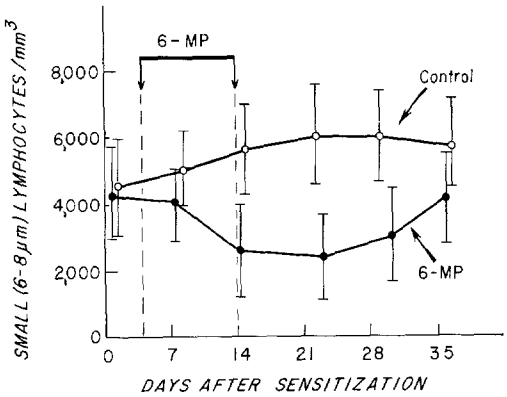

FIG. 3. Effect of 6-MP administration on the circulating small lymphocyte level. Significant although less striking suppression of total small lymphocyte count was found in group A (●) animals.

Cell Transfer Studies.—

Transfer of normal cells to group A animals: Selected animals of group A were utilized as recipients in cell transfer studies. Tuberculin skin tests were performed 7 days after cessation of 6-MP treatment; the mean response was less

than 2-mm diameter induration. Recipient animals were injected intravenously with either: (*a*) 1.5–2.0 × 10^8 bone marrow cells, (*b*) 1.5–2.0 × 10^8 glass nonadherent lymph node cells, or (*c*) Hanks' solution, used as a control. Normal, unsensitized guinea pigs served as donors for the pooled marrow or node cells. Tuberculin skin tests were then repeated in recipients 18 h after transfer (Table II).

Group A animals receiving bone marrow cells showed a significantly greater subsequent skin test response to tuberculin compared with pretransfer responses. Animals receiving equivalent numbers of bone marrow cells, which had been previously passed over glass bead columns to remove adherent cells, showed a lesser increase in tuberculin skin test reactivity. Group A recipients of

TABLE II

Effect of Normal Cell Transfer into 6-MP-Treated Guinea Pigs on Day 21 after CFA Administration

Cell source	Animals per group	Mean number and range of transferred cells	Skin test*	
			Before	After
		× 10^7		*mm*
Control: Hanks' solution	6	0	<2	<2
Nonadherent lymph node cells	6	7.5 (5–10)	<2	<2
Bone marrow cells	9	7.5 (5–10)	<2	9.7 ± 2.6
Nonadherent bone marrow cells	4	7.5 (5–10)	<2	3.4 ± 1.7

* Skin test reactivity (mean diameter of induration) to 1 μg of PPD (Weybridge) injected before and 18 h after cell transfer.

node cells showed no significant change in skin test responses. In limited experiments, responses of group A animals to intradermal injection of turpentine-oil irritant were compared before and after receipt of the normal cells. These responses changed in parallel with that to tuberculin, although to a lesser extent. As a control, group B or normal, unsensitized recipients were infused with Hanks' solution, bone marrow, or lymph node cells in a manner similar to that described above. No significant change in reactivity to tuberculin or irritant was noted.

Transfer of sensitized animal cells to normal recipients: Peritoneal exudate cells were obtained on days 22–24 from group A animals or group B animals. These cells were washed, pooled within a group, and injected intravenously in amounts of 1.5 × 10^8 into normal, unsensitized guinea pigs (Table III). Skin testing 18 h later demonstrated that in 7 of 11 cases peritoneal cell populations were able to transfer skin test reactivity to unsensitized recipients despite the

lack of skin test reactivity in the group A donors. However, it should be noted that the number of peritoneal exudate cells utilized in these transfers was not adjusted for the fact that the exudate subpopulations of cells were different in group A and group B animals.

These in vivo studies suggested that a primary site of action of 6-MP in suppressing delayed hypersensitivity was on the monocyte-macrophage population; its effects on the lymphoid cells were much less. Therefore, it was important to explore the functional activity of the lymphocytes with in vitro techniques allowing for more quantitative measurements.

In Vitro Lymphocyte Proliferative Responses.—Fig. 4 shows the tuberculin-induced proliferative response, expressed as an isotope incorporation index of

TABLE III

Effect of Transfer of Cells from Sensitized Animals to Normal Recipients

Cell source	Recipient skin test reactivity*		Donor skin test reactivity‡
	Before	24 h after	
		mm	*mm*
Peritoneal exudate cells from A donors (N = 11)	<2	6.9 ± 2.7	2
Peritoneal exudate cells from B donors (N = 11)	<2	9.3 ± 3.1	7.5 ± 1.9

* Skin test reactivity (mean diameter of induration) to 1 μg of PPD (Weybridge) injected before and 24 h after transfer of 1.5×10^8 peritoneal exudate cells.

‡ Skin test reactivity of peritoneal exudate cell donor.

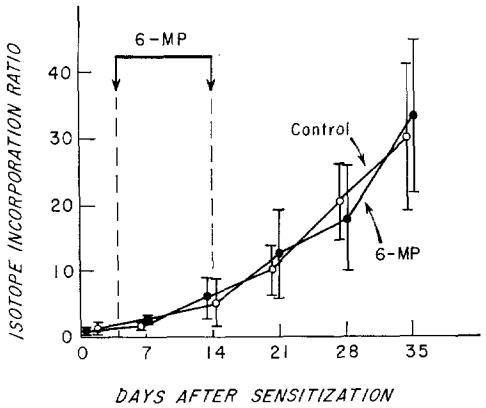

FIG. 4. Effect of in vivo 6-MP administration upon in vitro proliferative response to PPD. Lymphocytes from guinea pigs, sensitized to CFA and treated with 6-MP or placebo, were tested at weekly intervals for their response in vitro to Koch's OT. No difference was observed between the two groups. The results are expressed as an I.I.I., as described under Materials and Method.

lymphocytes obtained weekly from animals of groups A and B after the injection of CFA. It can be seen that there were no differences in the mean responses, even though the lymphocytes were obtained during periods of marked suppression of tuberculin skin reactivity in animals of group A. In addition, no difference in the mean response to phytohemagglutinin was noted between the two groups. This similarity in proliferative responses, measured by scintillation spectrometry, was confirmed by autoradiographic studies of isotope incorporation into DNA and the estimation of the prevalence of lymphocytes undergoing blastoid transformation and cell division (Table IV).

TABLE IV

Effect of 6-MP Administered In Vivo on In Vitro Proliferative Response to PHA and OT

Group*	In vitro mitogen	I.I.I.	Labeled‡	Blast§	Mitotic index‖
			%	%	
A	None	–	1.1	9	1.3
	PHA	51	27	82	34
	OT	17	13	44	24
B	None	–	0.6	11	0.4
	PHA	47	23	86	32
	OT	21	11	49	27
Uns.	None	–	1.3	5	0.8
	PHA	59	32	92	38
	OT	1.2	1.6	7	1.0

* A: cells derived from animals sensitized to CFA and treated with 6-MP; B: cells derived from animals sensitized to CFA and treated with NaOH diluent; Uns.: cells derived from normal, unsensitized guinea pigs.

‡ Percent of cells labeled with [^{3}H]Tdr by autoradiography.

§ Percent of cells undergoing morphologic blast cell transformation.

‖ Number of cells in metaphase/1,000 cells counted.

Production of Migration Inhibition Factor.—The effect of tuberculin in the in vitro migration of macrophages obtained from animals in groups A and B was first studied by the direct inhibition technique. Peritoneal exudate cells obtained from animals in groups A and B were assayed for migratory rate in individual chambers with and without the presence of tuberculin. There was no significant difference in the percentage of animals in each group whose peritoneal cells were markedly inhibited in this activity by the presence of tuberculin (Table V). In addition, the mean degree of inhibition produced by tuberculin was similar for the cells obtained from both groups.

However, it was observed that the mean migratory rate of the peritoneal exudate cells from group A animals in control chambers containing medium without tuberculin was significantly less than that of cells obtained from group B animals in the same medium. These peritoneal cells have been shown (16) to

be a heterogeneous group consisting mainly of macrophages, with a minority of lymphocytes of various sizes and small numbers of cells of other types. Therefore, the question arose as to whether the decreased migration rate was due to lymphocyte action or intrinsic macrophage limitations.

The first possible explanation was that the lymphocytes present in the peritoneal cell collection obtained from group A animals spontaneously liberated excess MIF activity or some cytotoxic factor.

TABLE V

Macrophage Inhibition Factor Activity

		Direct Assay		
Group*	Tested	Positive‡	Inhibition	
			%	
A	25	21	84 ± 12	
B	26	23	87 ± 9	
		Indirect Assay		
Group§	Tested	Positive‖	Inhibition¶	Inhibition**
			%	%
A	12	10	80 ± 13	19 ± 13
B	11	9	83 ± 11	13 ± 12

* A: peritoneal exudate cells from animals injected with CFA and treated with 6-MP; B: peritoneal exudate cells from animals injected with CFA and treated with NaOH control solution.

‡ Positive defined as PPD-induced inhibition of migration ≧30% as compared with migration in Ag-free medium.

§ A: MIF produced by lymphocytes of group A animals; B: MIF produced by lymphocytes of group B animals.

‖ Defined as inhibition of migration ≧30% (see text).

¶ Percentage inhibition of normal guinea pig exudate cells in the presence of MIF.

** Percentage inhibition of normal guinea pig exudate cells after MIF had been removed and replaced with fresh medium.

Therefore, the initial investigations centered on a comparison of the appearance of MIF activity in the supernatants of lymphocyte cultures derived from animals from groups A and B in the presence and absence of tuberculin. The patterns of macrophage migration inhibitory activity in the supernatants were similar when the lymphocytes from groups A and B were incubated with tuberculin (Table V). The migration rate of such peritoneal cells from unsensitized animals was then compared after the supernatant had been removed. The cells regained normal migratory capacity. Therefore, one could not explain the depressed migratory rate of peritoneal cells obtained from group A animals on the basis of the elaboration of a cytotoxic substance. In some experiments,

serial dilutions were made of the supernatants obtained when lymphocytes from animals of groups A and B were incubated with tuberculin. In this way it was found that approximately equal amounts of MIF were elaborated by comparable numbers of lymphocytes from each group. There was no significant MIF activity in the supernatants when lymphocytes from either group were incubated in the absence of tuberculin. Therefore, the decreased spontaneous migratory rate of peritoneal exudate cells from group A animals could not be explained on the basis of spontaneous elaboration of MIF or cytotoxic activity by lymphocytes within that exudate population.

Macrophage-Monocyte Function.—Another possibility was that there was an alteration in the intrinsic macrophage function in the 6-MP-treated animals. Therefore, the activities of the peritoneal cells from groups A and B were studied

TABLE VI

Peritoneal Macrophage Studies

Group	Total macrophage* yield	Migratory rate	Phagocytosis‡	Adherence§	Viability‖
	$\times 10^6$	*mm/h*	*cpm* $\times 10^5$	%	%
A	16.91 ± 6.81	0.42 ± 0.24	1.2 ± 0.7	61 ± 14	97
B	83.3 ± 16.3	0.94 ± 0.29	3.7 ± 1.6	88 ± 8	98

* Defined by morphologic and phagocytic criteria.

‡ Defined as cpm of ^{14}C particles ingested per 10^5 macrophages.

§ Defined as $\frac{\text{number of macrophages added} - \text{number not adhering glass}}{\text{number macrophages added}} \times 100.$

‖ Defined by trypan blue exclusion and phase contrast criteria.

in a number of ways: a significantly lower number of morphologically defined macrophages could be obtained by lavage of the peritoneal cavities in group A compared with group B animals (Table VI) 72 h after intraperitoneal instillation of mineral oil. There was also a mean decrease of 57% in the number of recovered lymphocytes. Once obtained, the cells of group A animals were as viable as those of group B, but were noted to spread less well on glass and to have a less highly developed refractile internal structure. In addition, these cells showed significant impairment in several in vitro functions when compared with cells siilarly obtained from group B animals. These included (*a*) adherence to glass beads, (*b*) migration on glass surfaces, and (*c*) phagocytosis of ^{14}C-labeled particles. Phagocytosis was also measured by visual determination of the number of ingested latex particles per macrophage. Mean and standard deviation values were obtained in these studies (Fig. 5). They suggested that the suppressed phagocytic activity of cells from group A animals was a general phenomenon involving most, if not all, of the cells.

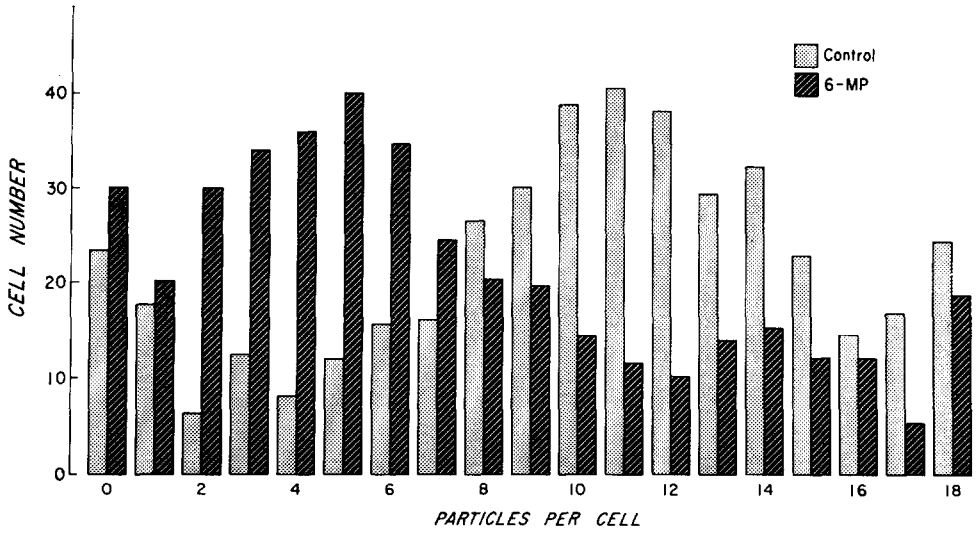

FIG. 5. Effect on in vivo administration of 6-MP upon subsequent phagocytosis by peritoneal macrophages in vitro. A general decrease involving most, if not all, cells was found with cells from 6-MP-treated animals.

DISCUSSION

The results of this study confirm previous findings (1) of the capacity of 6-MP to suppress the appearance of tuberculin skin test reactivity in the guinea pig. Current evidence from a number of studies suggests that the expression of the delayed hypersensitivity response requires at least two populations of mononuclear inflammatory cells. One cell type is a lymphocyte that has become specifically sensitized to the antigen in question. Such cells probably represent a small minority of the inflammatory cells at the skin test site (17). The majority of cells appear to be young, rapidly dividing, monocytic cells of bone marrow origin (18) that are recruited secondarily at the site of the interaction of antigen with sensitized lymphocytes by release of biologically active lymphokines (19). These studies are designed to study the specific and nonspecific cell populations during 6-MP-induced suppression of delayed hypersensitivity.

The results suggest that a major mechanism of 6-MP-induced suppression during developing delayed hypersensitivity is related to the drug's action on the "nonsensitized" cells of bone marrow origin. The following observations provide evidence for this conclusion: (*a*) The depression of blood monocyte levels was relatively greater than that seen in levels of small and medium lymphocytes during 6-MP treatment. (*b*) Sequential studies showed a striking suppressive effect of 6-MP on the marrow at a time when blood monocyte levels were depressed. (*c*) Animals with depressed tuberculin skin reactivity after 6-MP treatment exhibited suppressed skin reactivity to injected lymphokines. These

functionally defined substances are liberated from sensitized lymphocytes after in vitro interaction with a specific antigen (7). Intradermal injection of these supernatants has been reported (9) to induce a delayed hypersensitivity response characterized by infiltration of mononuclear cells. (*d*) Transfer of bone marrow cells from normal, unsensitized donors to animals in which tuberculin skin test reactivity had been suppressed after CFA and 6-MP treatment was followed by positive skin test responsiveness within 24 h. (*e*) In vitro measurements of macrophage function demonstrated decreased adherence to glass, migratory rate, and phagocytic capacity in the cells of the group A animals. Studies of the in vivo kinetics of this cell population will be reported later.

The data from this study also suggest that 6-MP, as utilized here, does not exert a striking effect on the development of a lymphoid cell population that becomes sensitized to tuberculin. (*a*) There was only a very modest suppression of the level of circulating small lymphocytes compared with the observed effects on monocyte levels. This difference in drug effect on the two cell types was reflected in a relative increase in the percentage of lymphocytes in the mineral-oil-induced peritoneal exudates obtained from 6-MP-treated animals compared with exudates from untreated animals. In addition, there was no significant effect on the morphology of the lymph nodes, in contrast to the marked depletion of the monocyte population in the bone marrow. (*b*) In at least some instances, peritoneal exudate cells from 6-MP-treated animals with suppressed tuberculin delayed hypersensitivity could transfer such reactivity to normal, unsensitized recipients. (*c*) As noted above, bone marrow cells from normal, unsensitized donors could reconstitute delayed hypersensitivity in animals in which this was suppressed by 6-MP treatment. Since expression of such skin test reactivity probably requires the presence of a small population of lymphoid cells sensitized to the antigen, one may assume that such cells were present in the 6-MP-treated animals, even though skin test reactivity was markedly suppressed. (*d*) Lymphoid cells from 6-MP-treated animals incubated with antigen in vitro reacted in a manner similar to that of cells from animals given the placebo solution. There was a similar sequential development of proliferative responsiveness of lymphocytes to tuberculin. This pattern had previously been observed in our laboratory (20) when lymphocytes of animals receiving CFA without any additional therapy were used, and is thought, as reviewed by Ling (21), to indicate development of cells immunologically committed to tuberculin. In addition, lymphocyte responses to a nonspecific mitogen, phytohemagglutinin, were not affected. This finding was evidence against nonspecific in vivo impairment of lymphocyte reactivity. (*e*) In vitro incubation with tuberculin led to a similar release of migration inhibition factor by lymphocytes obtained from both 6-MP-treated and placebo-treated animals on days 21–24 when tuberculin skin test reactivity was markedly suppressed in those animals previously treated with 6-MP. As 6-MP treatment had been discontinued for 1 wk, all the 6-MP should have been metabolized in vivo (22). There was no evi-

dence of nonspecific release of MIF or of cytotoxic factors by these cells. At this time the presence of tuberculin led to similar degrees of inhibition in the migration of peritoneal cells from both groups of animals in the direct MIF activity assay.

The findings of this study can be considered in relationship to previous investigations of the mechanisms of 6-MP action. The anti-inflammatory effect observed here was similar to that noted by Page et al. (6) and Hurd and Ziff (23) when irritant suspensions were used. The pattern of suppression of delayed hypersensitivity in the present study was also similar to that noted by Hoyer et al. (1).

A major effect of 6-MP is to reduce the number of mature monocytes that are able to participate actively in skin inflammatory reactions. This effect can be very prominent in the face of the known short half-life of such cells (24) and the findings by McCluskey et al. (17) that most cells in the delayed hypersensitivity skin test reaction are rapidly dividing ones. Hurd and Ziff (23) observed that the depressive effect of 6-MP on cellular nonspecific inflammation correlated best with the decrease in circulating monocytes and the depression of rapidly dividing mononuclear cells in the dermal inflammatory response. Delayed skin test reactivity is suppressed for only 1–4 wk after discontinuation of 6-MP treatment. In addition, 6-MP given for a period of time before, but not after, the sensitization injections does not lead to subsequent suppression of skin test reactivity (25). The early stages of the immunologic response in experimental allergic thyroiditis are less sensitive to 6-MP treatment that the later stages, after the onset of clinical disease (26). This mechanism of drug action may explain the clinical observation that 6-MP-induced immunosuppression is dependent on continuous drug administration.

However, other studies have indicated that the effects of 6-MP on cellular immunity depend on the timing of 6-MP administration relative to antigenic stimulation. Recent evidence (27) has suggested that the critical period of antigen recognition may occur within 48 h after primary exposure of the subject to that antigen. Borel and Schwartz (25) found that immunologic nonreactivity to a specific antigen could be induced in rabbits by 6-MP administration. They concluded that, under certain experimental conditions, the inhibition of expression of delayed hypersensitivity in drug-treated animals was accompanied by the inhibition of development of sensitized lymphoid cells to the specific antigen as well as by an anti-inflammatory effect.

Although the evidence in the present study did not suggest any significant effect on the development of tuberculin-sensitized lymphocytes, it may be questioned whether the administration of the drug sooner after the injection of CFA may have been associated with such a suppressive effect. The protocol of the present study was specifically designed to assay the effects of 6-MP started at a later time. Studies of alternative modes of drug administration are now in

progress in this laboratory, in an attempt to define further the mechanisms of 6-MP activity.

SUMMARY

The mechanism of suppression of delayed hypersensitivity to tuberculoprotein by 6-mercaptopurine (6-MP) was studied in guinea pigs. Under the conditions of the protocol, suppression of tuberculin delayed skin test reactivity was not associated with a significantly altered end-organ response to mediators of permeability. No significant alteration of in vivo lymphoid activity, as measured by reconstitution studies, was found. In addition, lymphoid cells from 6-MP-treated animals reacted in a fashion similar to those of placebo-treated animals with respect to (*a*) antigen-induced lymphocyte proliferation, (*b*) antigen-induced liberation of macrophage inhibitory factor activity, (*c*) direct inhibition by antigen of peritoneal exudate cell migration.

Conversely, suppression was seen in levels of blood monocytes and in vitro function of macrophages from 6-MP-treated animals in several respects: (*a*) adherence to glass, (*b*) migratory rate, (*c*) phagocytic capacity. Therefore, it would appear that a major mechanism of 6-MP-induced suppression of delayed hypersensitivity is through its action on effector cells.

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