

The Effect of 6-Mercaptopurine on the Inflammatory Response Stimulated by Subcutaneous Implantation of Polyvinyl Sponge

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A VARIETY of pharmacological agents have been shown to interfere with the evolution of the inflammatory response in animals and man. For any one of these compounds, the mechanism of action remains uncertain, except within the restrictions of the experimental system in which its biological effects have been investigated. In experimental studies it has frequently been assumed that the metabolic and anti-inflammatory activity of an agent were closely interrelated.^{1,2} In spite of this the most potent and most extensively studied anti-inflammatory agents, the glucocorticoids, have several well-defined metabolic actions, but the mechanism by which they suppress inflammation remains obscure.^{1,2}

During the past 15 years the antimetabolic effects of the drug 6-mercaptopurine (6-MP) have been assessed clinically and experimentally.^{3,4} In 1958 Schwartz, Stack, and Dameshek reported that the pharmacological actions of this drug included inhibition of antibody production in experimental animals.⁵ Many subsequent observations have further defined this "immunosuppressive" effect.^{3,4,6} It has recently been recognized that 6-MP would modify

the inflammatory response, and that this purine antagonist had anti-inflammatory activity. This action was shown to be independent of the biological effects of this agent on immune mechanisms.⁷⁻¹⁰

In the current study subcutaneous implantation of polyvinyl sponge in guinea pigs has been employed to stimulate inflammatory ingrowth of new-formed connective tissue. It was found that during administration of 6-MP the histology was altered, with suppression of the normally occurring foreign body giant cell response, dysplasia of collagen fiber deposition, and defective formation of capillary channels. The morphologic findings correlated with chemical evidence of a decrease in tissue content of desoxyribose, phospholipids, an increase in the content of adenine and non-collagenous proteins within the granuloma. These observations furnish additional evidence that the cells involved in a chronic inflammatory response are susceptible to the antimetabolic actions of 6-mercaptopurine.

METHODS

The technique of implantation and removal of sponge granulomas from adult mongrel guinea

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Table 1.—Dose, Duration of Treatment, and Vehicle Used in Administration of 6-MP to Guinea Pigs Bearing Polyvinyl Sponge Granulomas

Vehicle	Group designation	Dose and days treated 6-MP, mg./kg./day				Number of animals
		50	25	10	0	
O.25N NaOH	Na-6MP 50	1-7	8-42			5
O.25N NaOH	Na-6MP 25		7-42			30
Methylcellulose	MC-6MP 25		1-14			5
Methylcellulose	MC-6MP 10			7-42		5
O.25N NaOH	Control				1-4	6
Methylcellulose	Control				7-42	6
Saline	Control				7-42	6
No Injections	Control				7-42	12

pigs was carried out as previously described.¹¹ Ascorbic acid was added to the drinking water for at least 5 days post-implantation. Body weight was recorded on each animal every third day. Sponge implants were removed at 1, 4, 7, 10, 14, 17, 21, 28, 35 and 42 days. Peripheral white blood cell counts, films, and hemoglobin determinations were accomplished by standard laboratory methods. Gross autopsy examination was carried out on each animal.

6-Mercaptopurine (purinethol) was kindly supplied by Burroughs Wellcome and Company, Tuckahoe, New York. As suggested by this manufacturer it was dissolved in alkali (150 mg./ml. in 0.25 N NaOH, final pH 9.5), prepared fresh on alternate days, and stored at 4° C. It was administered by intraperitoneal injection. The drug was also suspended in 0.25 per cent methylcellulose (50 mg./ml.), and in one group of animals 6-MP in alkali was neutralized by stepwise addition of HCl prior to injection. The 4 experimental groups are as indicated in Table 1. Control animals received intraperitoneal injections of 0.25 N NaOH, 0.25 per cent methylcellulose, physiologic saline, or no injections.

Histological studies were carried out on 1 of the 4 implants removed from each of the animals.¹¹ Multiple sections were prepared from each tissue block, and tissue penetration into the sponge prosthesis measured at 3 positions on the tissue cross-sections using a linear ocular micrometer. Enumeration of the number of foreign body giant cells was carried out under X125 magnification using a rectangular grid with a total area of 0.4 mm.² At least 2 subcapsular and 2 central fields selected at random were counted in each tissue preparation.

The wet weight of the other 3 sponge implants from each animal was determined, and the net tissue wet weight was established by subtraction of the pre-implant weight of the dry sponge

prostheses. One aliquot was homogenized and extracted with 10 per cent trichloroacetic acid at 4 C. Following filtration and removal of acid with diethyl ether, this extract was analyzed for adenine and acid soluble protein content.¹² The rest of the tissue was cut into small pieces and dehydrated *in vacuo* over phosphorus pentoxide to constant dry weight. Lipids were extracted with ethanol-ether 3:1 (v/v), re-extracted into chloroform, and cholesterol, phospholipid, and total lipid content determined.¹³ The tissue solids remaining after extraction with ethanol-ether were dried and ground in a Wiley mill, and weighed aliquots were analyzed for total protein by a micro-Kjeldahl procedure.¹⁴ Desoxyribose content was determined on hot trichloroacetic acid supernatant of other samples by the diphenylamine reaction.¹⁴ Five and 10 mg. aliquots were hydrolyzed with 6 N HCl in sealed glass tubes for 20 hours and analyzed for hydroxyproline by the Leach modification of the method of Newman and Logan.¹⁵

RESULTS

Systematic Response. As noted in Table 1, control animals that received unbuffered 0.25 N NaOH succumbed within 1 to 4 days to chemical peritonitis. The other control animals tolerated 0.25 per cent methylcellulose or saline, maintained or gained weight, and had normal peripheral blood counts. Drug toxicity was observed in the animals that received daily injections of 6-MP suspended in methylcellulose (MC-6MP 25). In this group the drug was poorly tolerated, and progressive weight loss occurred as well as persistent suppression of the bone marrow. Administration of drug to

these animals was limited to a total of 14 days, and the longest surviving animal yielded granulomas 17 days of age. Administration of 6-MP in alkali (Na-6MP 25, Na-6MP 50) or 0.25 per cent methylcellulose (MC-6MP 10) was tolerated without overt signs of toxicity. Drug treatment of these animals was modified after 3 weeks according to the level of the peripheral white blood cell count. Frequency of injection was reduced to every second or third day for the duration of the individual experiments.

A relatively persistent leukocytosis in the peripheral blood was noted in control animals (Fig. 1) subsequent to sponge implantation. This was uniformly suppressed in all drug treated animals and was dose-related. Qualitative examination of peripheral blood films demonstrated suppression of platelets. There was a tendency toward normalization of the total peripheral white blood cell count after 21 days, with the reduction in frequency of 6-MP injections. In the animals treated for 21 to 42 days, hemoglobin concentrations generally showed a progressive decrease to the range of 7-10 gm./100 ml.

Histological Studies. During the first 3 days post-implantation, "wound fluid" filled the sponge, and there were significant numbers of polymorphonuclear leukocytes and hematogenous mononuclear cells present within the interstices of the implant. In control implants the anticipated pattern of penetration and organization of the sponge prosthesis by young inflammatory connective tissue was observed between 3 and 42 days post-implantation.¹¹

Irrespective of the degree of suppression of the peripheral total white count during 6-MP administration, there was no evidence of alteration in the early exudative response within the sponge implant between 1-7 days. However, on examination of serial cross sections 7-42 days of age, there was histological evidence that the drug had

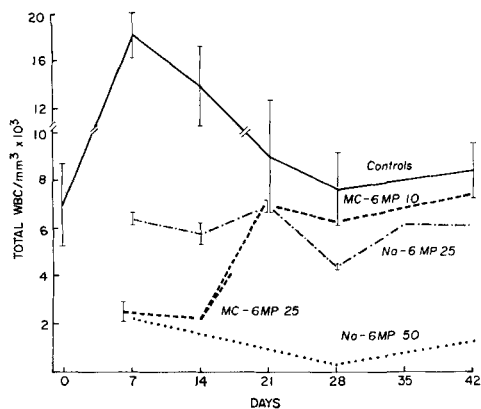


Fig. 1.—Response of guinea pig total peripheral white blood cell count to polyvinyl sponge implantation in control animals, and in those receiving intraperitoneal 6-MP in methylcellulose (MC-6MP 10 mg., MC-6MP 25 mg.) or alkali (Na-6MP 25 mg., Na-6MP 50 mg.).

modified connective tissue formation. In Table 2 the depth of tissue penetration, expressed as a fraction of the total cross section of the sponge, is compared for control and drug treated animals. Retardation of connective tissue organization of the sponge implant related to the dose of 6-MP was found at 7-17 days. Delay in organization of the sponge prosthesis was also present in all animals treated for 35-42 days. Between 21-28 days, penetration was not uniformly suppressed in the animals that received 6-MP. The histological findings are illustrated in Fig 2a and b, where connective tissue organization at 21 days is compared for a methylcellulose injected control animal and one that received 10 mg. MC-6MP for 35 days.

Foreign body giant cells were normally present in granulomas in increasing numbers after 7-10 days, and were a prominent part of the host response to subcutaneous polyvinyl sponge implantation. In Table 2 the mean difference in the number of foreign body giant cells per unit area is compared for all control and drug treated animals. Suppression of foreign body giant

Table 2.—Comparison of Extent of Connective Tissue Organization of the Polyvinyl Sponge Implant, and Number of Foreign Body Giant Cells in Control and 6-MP Treated Animals

Tissue age in days	Penetration *				FBGC † per 0.4 mm ²	
	Controls	Na — 6—MP 25 & 50 mg.	MC — 6—MP 10 mg. 25 mg.		Controls	6—MP
7	.15 ± .05 ‡	.03	.05	.04	0.6	0.5
10	.28			.13	2.9	0.6
14	.46 ± .04	.31	.42	.11	7.1	3.0
17				.15		2.0
21	.70 ± .10	.66	.77		7.5	4.9
28	.90 ± .09	.88	.94		8.0	2.6
35	.94	.79	.67		8.4	3.7
42	.98 ± .02	.76	.88		8.0	5.1

* Fraction of microscopic cross section area invaded by new-formed connective tissue.

† Foreign body giant cell.

‡ Mean and range of observed values.

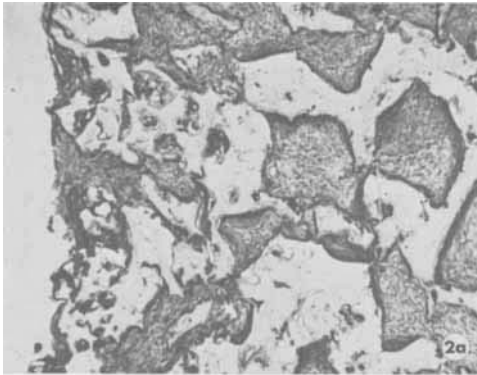


Fig. 2a.—Low-power view demonstrating the depth of penetration of polyvinyl sponge by new-formed connective tissue in a 21 day control animal. Capsular margin is at the left, and center of cross section at the right. Azure A stain ($\times 34$).

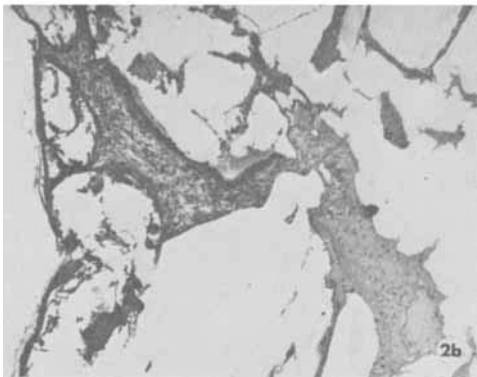


Fig. 2b.—An example of suppression of normal connective organization of the sponge implant after 35 days of treatment with 6-MP in methylcellulose, 10 mg./kg./day. Orientation is the same as in Fig. 2a. Note unorganized "wound fluid" in the deeper sponge interspaces (lower right of figure). Azure A stain ($\times 34$).

cell response was found at each tissue age and in all of the animals that received 6-MP, irrespective of the daily dose of drug (Fig. 3a and b). Treatment with 6-MP did not alter the morphological appearance of these cells or their tendency to inter-

pose themselves between the sponge prosthesis and the rest of the tissue within an interspace.

Defective formation of capillary channels during tissue organization of the sponge implant was present in sponge granulomas

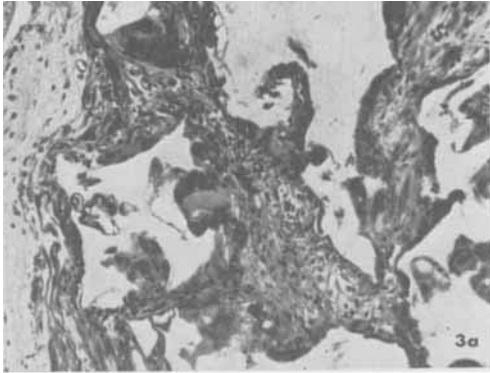


Fig. 3a.—Subcapsular area of a 21 day control sponge implant showing the normal foreign body giant cell response. Masson trichrome stain ($\times 133$).

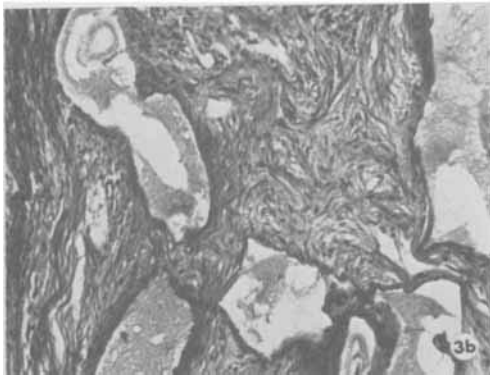


Fig. 3b.—Subcapsular area of a 21 day sponge implant removed from an animal that had received 6-MP in alkali, 25 mg./kg./day. A single foreign body giant cell is present at the lower right of this section. Masson trichrome stain ($\times 133$).

removed from drug treated animals. As illustrated in Fig. 4b, focal areas of hemorrhage were found at the sites of active invasion of the sponge by new-formed connective tissue in granulomas 7-42 days of age. In control implants a hemorrhagic appearance was grossly apparent from 1-7 days post-implantation. During 6-MP administration this gross finding persisted in many instances for 21-28 days. Since this was noted prior to removal of implants from the pseudocapsule, and since this tissue was far less adherent in 6-MP treated animals, microscopic evidence of interstitial hemorrhage could not be attributed to the technique used in removing the sponge granulomas. Depression in blood platelet concentration undoubtedly contributed in some degree to the hemorrhagic tendency. However, clear evidence of a defect in angioblastic activity resulting in impaired formation of new capillaries was

always observed microscopically (Fig. 4b).

During normal connective tissue organization of the sponge, deposition of dense bands of collagen fibers occurred at the outer margin of each sponge interspace. In animals treated for more than 10 days with 6-MP 25 mg./kg./day, an unusual histological pattern of collagen fiber formation was observed. In these granulomas (Fig. 5b) there was evidence of a random, disorganized deposition of what appeared histologically to be increased amounts of collagen. In tissue sections stained with the Masson trichrome technique, tinctorial characteristics of the collagen fibers were normal. This change in histology of the tissue was unaccompanied by any alteration in the morphology of the fibroblasts or variation in extracellular metachromasia. The number of tissue macrophages, undifferentiated mononuclear cells, and fibroblasts present in the 7-42

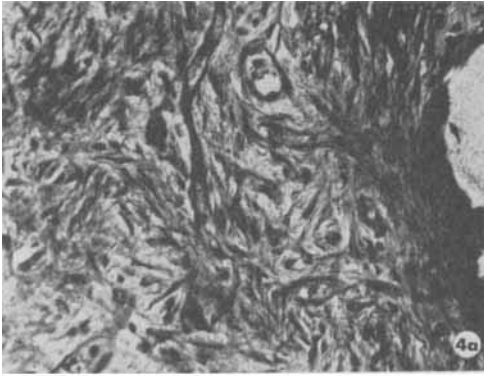


Fig. 4a.—High power view of a portion of a sponge interspace at 14 days near the advancing edge of connective tissue organization within an implant. The normal pattern of formation of several endothelial lined capillary channels is demonstrated. Masson trichrome stain ($\times 224$).

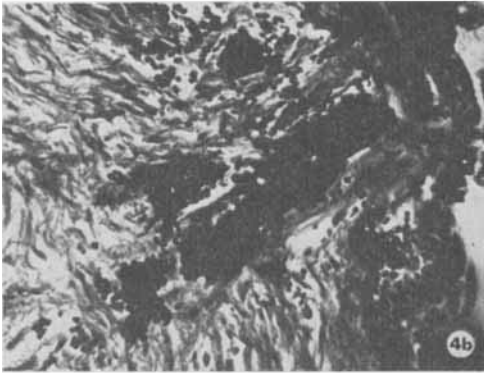


Fig. 4b.—An example of hemorrhage into the interstitial portions of an organizing interspace, selected from an area comparable to that in Fig. 4a. Angioblastic activity has been arrested and normal formation of capillary channels is absent. This granuloma was from an animal treated for 14 days with 6-MP in methylcellulose, 10 mg./kg./day. Masson trichrome stain ($\times 224$).

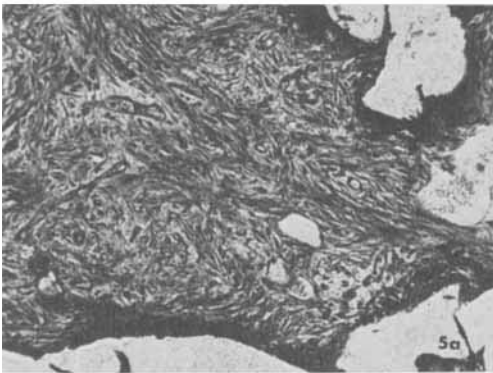


Fig. 5a.—Normal histological appearance of a sponge interspace at 21 days after complete penetration by connective tissue. Note peripheral location of deeply stained collagen fiber bundles and foreign body giant cells. Masson trichrome stain ($\times 112$).

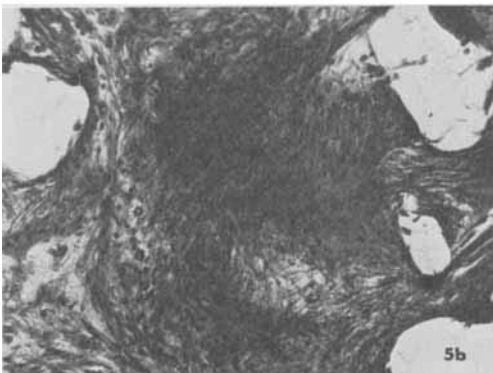


Fig. 5b.—An example of disordered collagen fiber deposition extending throughout a sponge interspace which was observed to some degree in all animals treated with 6-MP. The dysplasia in the normal pattern of peripheral deposition of dense bands of collagen in this sponge interspace was produced by treatment with 6-MP in alkali (25 mg./kg./day) for 21 days. Masson trichrome stain ($\times 112$).

day granulomas appeared to be unaffected by treatment with 6-MP.

Chemical Studies. Appropriate extracts were prepared from 3 of the sponge implants removed from each animal, and several of the chemical analysis correlated with the morphologic aberrations noted on histological examination of the tissue. An increase in tissue wet weight and net tissue solids was found in all but 2 instances for the drug treated granulomas (Table 3). This was not due to an increase in water content since net tissue dry weight, reflecting a true increase in total tissue solids, was also observed.

The increase in total tissue solids in the 6-MP granulomas resulted from an increase in tissue concentration of total protein (Fig. 6a). Hydroxyproline content as an index of the tissue concentration of collagen (Fig. 6b) was normal in all but one group of animals and did not contribute to the observed increase in total proteins. In the animals that received MC-6MP 25 mg./kg./day, increased collagen content per gram wet weight was found between 10 and 17 days. In these 4 animals, histologic evidence of the most marked aberration in normal collagen fiber deposition was noted. Hydroxyproline was undetected in unhydrolyzed aliquots of tissue, which indicated that measurable amounts of free hydroxyproline did not contribute to the analyses.

The observed increase in noncollagenous proteins noted in 6-MP-treated granulomas correlated with the histological evidence of defective capillary formation in implants 10-42 days of age. This finding suggested that proteins derived from the plasma were major contributors to the increase in total proteins in the developing granuloma.

The concentration of desoxyribose, as a measure of total desoxyribonucleic acid content, was depressed in all drug treated animals (Fig. 7a). This chemical evidence for decrease in cellularity in the implants

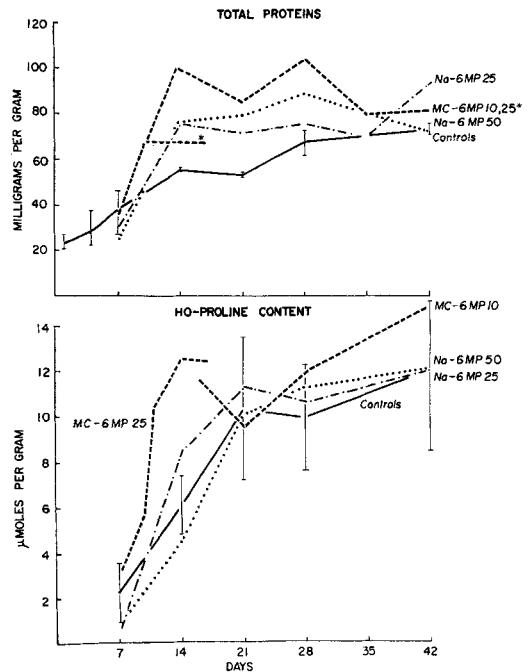


Fig. 6.—Changes in total protein and hydroxyproline (collagen) content in the polyvinyl sponge granuloma during administration of 6-MP in methylcellulose (MC-6MP 10 mg., MC-6MP 25 mg.) or alkali (Na-6MP 25 mg., Na-6MP 50 mg.).

was generally dose-related from 7-21 days. In the older granulomas reduction in frequency of drug treatment may explain the reciprocal change in desoxyribose content for Na-6MP 50, 25 mg. and MC-6MP 10 mg. animals after 21 days. The chemical analyses indicated that reduction in total cell numbers probably involved all major cell types within the granuloma, although microscopic examination identified only the foreign body giant cell as a prominent contributor to this change. The concentration of tissue phospholipids was similarly reduced in the granulomas removed from treated animals (Fig. 7b). Cell membranes have a high content of these substances, and the decreased amounts that were found contributed additional chemical evidence for a reduction in total cell population in the sponge implant. A variable decrease in

Table 3.—The Effect of 6-MP on Wet Weight and Total Tissue Solids in the Polyvinyl Sponge Granuloma

Tissue age in days	Controls		Wet weight, grams			Dry weight, milligrams				
			Na — 6-MP		MC — 6-MP	Na — 6-MP		MC — 6-MP		
			25 mg.	50 mg.		25 mg.	50 mg.			
7	1.16 ± .01 *	1.03 ± .03	1.55	0.97	1.28	93 ± 14	53 ± 8	27	95	109
10					1.19					126
14	1.15 ± .27	1.34 ± .07	1.64	1.18	0.96	103 ± 7	166 ± 30	212	165	
17										
21	1.05 ± .13	1.18 ± .05	1.21	1.10		106 ± 2	115 ± 13	147	151	
28	0.86 ± .02	1.06 ± .01	1.12	0.94		93 ± 2	129 ± 10	156	103	
35		0.99		1.01			101		119	
42	0.79 ± .08	1.04	1.12	0.72		64 ± 13	141	153	60	

* Mean and range of observed values.

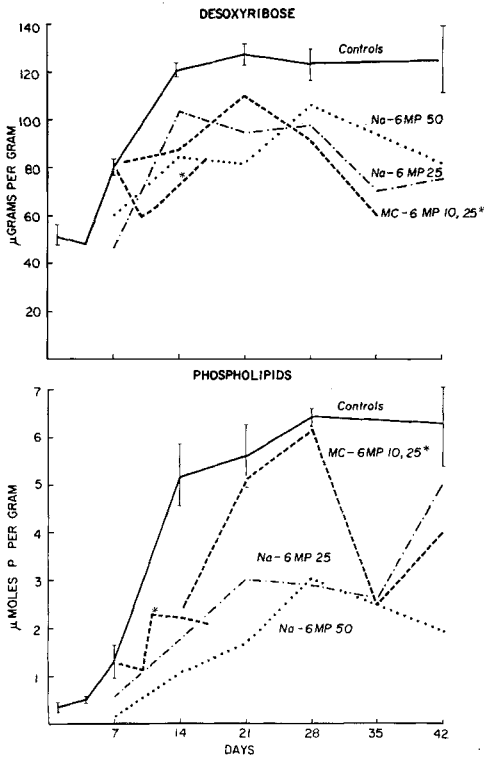


Fig. 7.—Reduction in desoxyribose and phospholipid concentration in polyvinyl sponge granulomas removed from animals treated with 6-MP in methylcellulose (MC-6MP 10 mg., 25 mg.) or alkali (Na-6MP 25 mg., 50 mg.).

tissue cholesterol was found. The observed reduction in total lipids was directly related to the decreased concentration in total tissue phospholipids.

The cold acid-soluble extracts of granulomas removed from 6-MP treated animals contained increased amounts of the purine base, adenine. Mean values were greater than 2.00 μ moles in 7-42 day drug treated (Na-6MP 25) and less than 1.25 μ moles per gram in all control granulomas. In contrast to the increase in total proteins (Fig. 6a), the age-dependent change in acid soluble protein content, 0.4 to 1.4 mg./gm., did not differ significantly in control and 6-MP treated granulomas.

DISCUSSION

The present study contributes additional histological and chemical evidence that certain of the biological effects of 6-mercaptopurine are anti-inflammatory in nature. The cells involved in proliferation of young inflammatory connective tissue share biological sensitivity to the effects of this agent with several other cell types.³⁻⁴ Reference has been made in previous studies to anti-inflammatory activity as a part of the pharmacological action of this antimetabolite. Suppression in the number of mononuclear cells present in inflammatory infiltrates in dermal connective tissue,⁷ and absence of joint inflammation in adjuvant-induced arthritis in rats,⁸ suggested that a dissociation between the apparent "immunosuppressive effect" of this agent and its anti-inflammatory action could be achieved. Schwab⁹ has demonstrated that chronic granuloma formation in rabbits stimulated by injection of streptococcal cell wall extracts was modified or suppressed during treatment with 6-MP. Recently Chamougan and Schwartz⁶ indicated that antibody formation could be stimulated or suppressed depending upon the time of administration of 6-MP, and one of these investigators also suggested that this drug had anti-inflammatory activity.¹⁰

As demonstrated in several studies^{3,6,16} designed to investigate the immunosuppressive action of 6-MP, significant variation in the results could be traced to differences in host susceptibility, suspending vehicle, route, or duration of administration of the drug. In the present investigation 2 vehicles and 3 dose levels were chosen for simultaneous comparison. It seemed logical to accept as biological expressions of the anti-inflammatory effect of 6-MP only those chemical and histological changes induced in the sponge granuloma in the absence of signs of host toxicity. In evaluating

the results it was apparent that an effect on cell numbers, antioblastic activity, and maturation of foreign body giant cells had occurred in the absence of toxicity. By contrast the interesting dysplasia in collagen fiber deposition and suppression of tissue organization of the implant were most marked in animals with signs of overt drug toxicity, although all of the changes observed in this investigation were present to some degree at each level of drug administration. It was also observed when the drug was administered in methylcellulose that the milligram per kilogram potency, as judged by most of the parameters studied, was greater than that found when the drug was given in alkaline or neutral solutions. It should be noted that the guinea pig has previously been found to be more resistant than several other experimental animals or man to a unit dose of 6-MP.¹⁶

To date the rationale for the use of 6-MP in selected patients with rheumatic disease has been to exploit the apparent immuno-

suppressive effect of this agent.^{3,17} Certain of the observed effects in patients with inflammatory disease appear to better correlate with recent experimental studies⁷⁻⁹ indicating that this antimetabolite has biological activity which can be defined as anti-inflammatory. The present investigation contributes additional evidence that the several cell types involved in the later stages of evolution of the inflammatory response demonstrate biochemical sensitivity to this antimetabolite. Appropriate use of this agent in experimental studies¹⁸ may assist in further elucidation of the factors important to the exquisite biochemical control of the inflammatory process.¹⁹

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SUMMARY

Administration of 6-mercaptopurine by intraperitoneal injection in methylcellulose or alkaline solution to guinea pigs caused: suppression of connective tissue organization of the subcutaneous polyvinyl sponge implant; a decrease in the number of foreign body giant cells present in the granuloma; a derangement in the normal formation of capillaries; and an unusual dysplasia in normal collagen fiber deposition. Several of these effects were dose-related. The morphological findings correlated with chemical evidence of a decrease in desoxyribose and tissue phospholipid concentrations. By chemical analysis an increase in total noncollagenous proteins was found during the period when histological evidence of defective capillary formation was most marked. In toxic doses the drug appeared to further accentuate the dysplasia in collagen fiber formation, and there was an increase in the amount of collagen in these granulomas. These findings support the concept that an important part of the biological activity of 6-MP can be defined as anti-inflammatory in nature.

SUMMARIO IN INTERLINGUA

In porcos de India le injection intraperitonee de 6-mercaptopurina in methylcellulosa o solution alcalin causava suppression del organisation de tissu conjunctive in subcutanee implantationes de spongia de polyvinyl, un declino in le numero de cellulas gigante de corpore alien presente in le granuloma, un disrangiamento in le formation normal de capillares, e un inusual dysplasia in normal depositos de fibra collagenic. Plures de iste effectos dependeva del dosage. Le constatationes morphologic esseva correlationate con evidentia chimic de un declino in le concentration de desoxyribosa

e de fosfolipido tissular. Per analyse chimic, un augmento esseva trovate in le total del proteinas noncollagenose durante le periodo quando evidentia histologic de un defective formation capillari esseva le plus marcate. A nivellos toxic de dosage, le pharmaco pareva accentuar additionally le dysplasia in le formation de fibra de collageno, e il occorreva un augmento in le quantitate de collageno in iste granulomas. Iste constatationes supporta le conception que un importante parte del activitate biologic de 6-mercaptopurina pote esser definite como anti-inflammatori in su natura.

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