EFFECT OF 6-MERCAPTOPURINE ON INFLAMMATION

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In 1958, Schwartz, Stack and Dameshek [1] first reported inhibition of antibody production with 6-mercaptopurine (6-MP), followed, in 1960, by Dameshek and Schwartz's [2] description of good results with clinical use of 6-MP in treating acquired hemolytic anemia, lupus erythematosus, and lupoid hepatitis. Encouraged by their experience, we used 6-MP in the treatment of two patients with plasma cell hepatitis and obtained remission of the liver disease in both cases. However, improvement did not begin until 3 to 4 weeks after the initiation of therapy, a lag difficult to explain on the basis of inhibition of antibody formation, since all available experimental evidence indicates that 6-MP has an immediate effect on antibody production.[3,4] This inconsistency prompted us to seek another biologic effect of 6-MP which might explain the inhibition of an inflammatory form of liver disease. The investigation took the form of a study of its effect on the cellular response to inflammation in rabbits, by means of Kolouch's connective tissue spread technique.[5]

MATERIAL AND METHODS

Rabbits

Albino rabbits of both sexes, weighing 1.5 to 2.5 kg., were obtained from a single local breeder and used in these studies. They were fed Purina rabbit pellets and offered water *ad libitum*. Preparation consisted of shaving the back with an electric clipper.

6-Mercaptopurine

Purinethol® brand 6-mercaptopurine was dissolved in 1 N NaOH at a concentration of 160 mg. per cc., and used within an hour of preparation because of the rapid loss of 6-MP's biologic activity on standing in this basic solution. Daily injections (0.03 cc. to 0.15 cc.) of the fresh preparation were given intravenously into the marginal ear vein. The solution had a pH of 10 and was only mildly irritating to the vein. Details of the dosage and duration of treatment are furnished in Table I.

Studies of Inflammatory Response

A 10 per cent solution of egg white was made in sterile 0.9 per cent saline. One drop of India ink was added to 2 cc. of this mixture. One tenth cc. of the resulting

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suspension was injected subcutaneously into each of 10 sites along the rabbit's shaved back. The time of injection in relation to pretreatment with 6-MP is shown in Table I, as is the leukocyte and differential count done in each animal on the day inflammation was initiated.

Biopsy specimens were obtained at 4, 8, 12, 24, 48, 72, and 96 hours after injection of the egg white, at a different site each time, using Kolouch's connective tissue biopsy technique.[5] A small incision was made in the skin over the injection site. Then the loose subcutaneous connective tissue in an area marked with India ink was picked up with fine-tooth thumb forceps and snipped off with scissors. Spreads of this tissue were made on clean glass slides, care being taken to obtain tissue samples which were one cell in thickness. The tissue was fixed by drying the slide rapidly in air and was then stained with a combination of Wright and Giemsa stains, as in staining blood or bone marrow films.

Experimental Controls

The normal inflammatory cycle is highly predictable in rabbits when a given irritant is used; both the types of cells at the inflammatory site and the time of their appearance are remarkably consistent from one occasion to another in the same animal and from animal to animal in groups of adult rabbits.[5,6] One group of controls for the present studies included 20 rabbits studied with identical methods on a prior occasion. Two additional biopsy specimens were taken, at 1 and 2 hours,* but otherwise the procedures were the same. A second small control group of 3 untreated animals was included in the present series; the results are shown in Table I. Further control data are available in the normal inflammatory cycles recorded in each dosage group during the early stages of 6-MP treatment (Table I).

Adrenalectomy

Bilateral adrenalectomy was carried out in 2 animals according to the method of Zak, Good and Good.[7] Postoperatively the animals were given as their only fluid source an electrolyte solution containing sodium, 150 mEq. per liter; chloride, 100 mEq. per liter; and bicarbonate, 50 mEq. per liter. They were given 12 mg. per kg. of 6-MP a day, beginning on the day following operation.

Classification of Anti-inflammatory Effect

In all instances the inflammatory responses were graded according to the following classification:

0, Animals in this group exhibited a normal inflammatory response.

+, Animals in this group showed a normal neutrophil response, but there was a delay in the appearance of mononuclear cells.

++, Animals in this group showed a normal neutrophil response, but there was both a delay in the appearance and a decrease in the intensity of the mononuclear response.

+++, Animals in this group showed a normal or slightly reduced neutrophil response, but the mononuclear cell response was either absent or greatly decreased.

++++, Animals in this group showed a complete lack of cellular infiltration.

RESULTS

The Normal Inflammatory Response

Following subcutaneous injection of egg albumin in the normal animal, an inflammatory cycle featured by an orderly sequence of events is

* Preliminary studies indicated that the 4-hour specimen provided the first significant indication of the effect of 6-MP on inflammation.

TABLE I

EFFECT OF VARIOUS 6-MP DOSAGES AND PRETREATMENT PERIODS ON INFLAMMATION IN THE RABBIT

6-Mercaptopurine (mg./kg./day)*	Days of pretreatment	Animal no.	White blood count (per cu. mm.)†	Neutrophils (per cent)†	Anti-inflammatory effect ‡
18	4	1	9,550	50	o
18	4	2	8,200	44	o
18	4	3	14,850	31	o
18	6	4	5,850	34	+
18	6	5	6,500	27	++
18	6	6	17,900	43	+
18	8	7	5,950	50	+++
18	8	8	12,800	36	+++
18	8	9	7,200	48	+++
12	o	10	11,450	17	o
12	o	11	6,850	30	o
12	o	12	4,550	38	o
12	4	13	8,350	43	o
12	4	14	14,400	74	o
12	4	15	7,350	39	o
12	6	16			+++
12	6	17			++
12	6	18			++
12	8	19	13,100	62	+++
12	8	20	12,000	47	+++
12	8	21	12,300	54	+++
12	12	22	18,800	51	+++
12	12	23	8,650	43	+++
12	12	24	8,650	60	o
12	12	25	19,750	84	+++
6	8	26	9,500	20	o
6	8	27	4,800	29	+
6	12	28	8,050	66	+
6	12	29	23,950	77	o
6	16	30	6,550	19	+++
6	16	31	4,600	48	++++
6	20	32	10,600	49	+++
6	20	33	1,950	26	++++ (died at 24 hr.)
6	28	34	21,500	34	+++
6	28	35	12,700	66	+++
3	20	36	8,700	64	o
3	20	37	7,000	55	o
3	30	38	17,000	57	o
3	30	39	14,200	50	+
3	40	41	19,250	62	+++
3	40	43	12,600	64	++
3	50	44	11,900	32	++
3	50	45	7,650	66	++++ (died at 24 hr.)
o		46	10,550	52	o
o		47	16,500	34	o
o		48	11,150	24	o

* Daily administration of 6-MP was continued until completion of the 96-hour biopsy.
† Peripheral blood counts performed on the day inflammation was initiated.
‡ Classification of anti-inflammatory response as given in text.

induced.[5,6] The typical inflammatory cycle in the normal rabbit (based on studies in 20 animals prior to the 6-MP series) was as follows. Within the first hour the connective tissue histiocytes underwent changes associated with their assumption of the role of active macrophages. Their cytoplasm became more basophilic, and ingested carbon particles (India ink) were seen within the individual cells. By 2 hours following injection, neutrophils were seen migrating into the inflammatory site. At 4 hours the first lymphocytes were seen in tissue adjacent to blood vessels. At the eighth hour sampling, lymphocytes were present throughout the field of inflammation, and these cells already appeared to be undergoing transformation into macrophages. Usually by 12 hours mononuclear cells, mostly of hematogenous origin, outnumbered the neutrophils and, from then on, became increasingly prominent. Dense mononuclear cell infiltration was seen at 24 hours and throughout the period of study (96 hours). Figure 1 illustrates the normal cycle at 4 and 48 hours. This is the response classified as "0" and was characteristic of all the untreated rabbits examined and of 6-MP-treated animals during the early period of treatment.

The Anti-inflammatory Effect

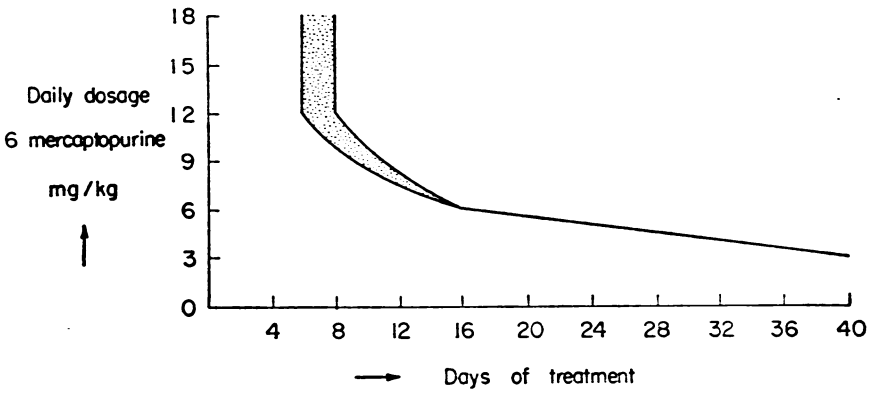
Although, as indicated above, there were degrees of anti-inflammatory effect (Table I), we have considered the response classified as "+++" to be typical (Fig. 2). Neutrophils appeared in normal or nearly normal numbers in the early specimens (at 4, 8, and 12 hours), but the hematogenous mononuclear cells, which normally appear in the 4-hour specimens, were not evident at that time. Thus, later biopsy specimens showed only histogenous macrophages containing ingested carbon, plus a few degenerating neutrophils. In some instances, small numbers of lymphocytes appeared in the 24 or 48 hour samples; this was unusual, however.

It is noteworthy that 3 animals had a response classified "++++," showing complete absence of both neutrophils and hematogenous mononuclear cells. Two of these animals died before the biopsy series was completed (Table I).

Dosage of 6-MP and Period of Pretreatment

Daily dosages of 12 mg. and 18 mg. per kg. had very similar effects (Table I). All animals pretreated for 4 days showed normal inflammation. However, those treated for 6 days exhibited a moderate anti-inflammatory effect (+ to ++), and 9 of 10 animals treated for 8 days showed a marked reduction in inflammation (+++). The single exception was a rabbit with a normal inflammatory response despite pretreatment with the 12 mg. dose for 12 days.

Rabbits pretreated with 6 mg. of 6-MP per kg. for 8 to 12 days showed normal or slightly decreased inflammation (0 to +); those given this dose for 16 days or longer exhibited marked reduction in inflammation (+++ to ++++). When the 3 mg. per kg. daily dose was given for 20 or 30 days, the animals showed normal or slightly reduced inflammatory responses (0 to +), but when treatment continued for 40 and 50 days, moderate to marked decreases (++ to ++++) became apparent. In Text-figure 1 a clear relationship between the 6-MP dosage and the period of treatment needed to achieve a marked anti-inflammatory effect (+++ or ++++) is indicated.



TEXT-FIGURE 1. Dose response curve illustrating the number of days of pretreatment with 6-MP required to inhibit inflammation.

Duration of Anti-inflammatory Effect

An attempt was made to determine the duration of the anti-inflammatory effect after discontinuing 6-MP in a small group of rabbits previously given 12 mg. per kg. daily for 12 days. Inflammation was initiated at the time of the last dose of 6-MP in 2 animals; examination of biopsy specimens revealed a marked reduction in inflammation throughout the 96 hours of observation. In 1 rabbit the inflammatory sites were prepared 24 hours after the last dose of 6-MP; the mononuclear response was delayed for 24 hours and appeared to be slightly reduced throughout the 96-hour period. Two animals given the inflammatory stimulus 48 hours after the last injection of 6-MP responded normally. Thus, it appears that the inhibitory effect of 6-MP on inflammation lasts between 24 and 48 hours after discontinuance of treatment.

Leukopenia and Weight Loss in 6-MP-treated Rabbits

The anti-inflammatory effect of 6-MP did not appear to be attributable to gross alterations in the peripheral blood leukocyte count (Table I). Only one rabbit developed leukopenia and neutropenia, although

more than 20 animals showed an anti-inflammatory effect. When 3 mg. per kg. was the daily dose, none of the animals developed a depressed count during the 40 and 50 days of pretreatment.

Animals treated with large doses of 6-MP lose weight during long-term experiments, and it has been suggested that the biologic effects of the drug might be secondary to debility and malnutrition.[8] Rabbits treated with 3 mg. per kg. per day gained weight over the period of treatment;

TABLE II

WEIGHTS OF RABBITS TREATED WITH 6-MP; DAILY DOSE, 3 MG. PER KG.

Animal no.	Initial wt. (kg.)	Weight (in kg.) at:			
		7 days	14 days	25 days	36 days
36	2.2	2.4	2.5	Sacrificed at 24 days	
37	1.8	1.9	2.0	Sacrificed at 24 days	
38	1.6	1.9	2.0	2.4	Sacrificed at 34 days
39	1.7	2.0	2.1	2.4	Sacrificed at 34 days
40	1.5	1.7	1.7	1.7	Died at 27 days
41	1.6	1.7	1.9	2.0	2.1
42	2.3	2.5	2.4	Died at 20 days	
43	1.7	1.9	2.1	2.0	1.8
44	1.9	2.1	2.4	2.4	2.5
45	2.2	2.4	2.5	2.4	2.5

they also seemed to thrive in every way (Table II). Although weight records were not kept on animals treated with the higher dosages of 6-MP for shorter periods during this experiment, subsequent studies have shown that rabbits tolerate 12 mg. per kg. of 6-MP for 8 days without weight loss.[9]

The Anti-inflammatory Effect in Adrenalectomized Animals

Since adrenal cortical hormones have heretofore been the most potent antiphlogistic agents known, the possibility existed that the anti-inflammatory activity of 6-MP in these experiments was secondary to adrenal stimulation. For this reason, an attempt was made to determine the effect of 6-MP pretreatment on the inflammatory response of adrenalectomized rabbits in a small pilot study. Of the 2 animals surviving adrenalectomy and treated with the 12 mg. per kg. daily dose of 6-MP, 1 survived for 12 days. The inflammatory response in this animal after

12 days of pretreatment revealed an intensive neutrophil infiltration, with complete lack of mononuclear cells at the inflammatory sites. Thus, the mononuclear response to an inflammatory stimulus was completely suppressed in a completely adrenalectomized animal, suggesting that the adrenal glands do not mediate the anti-inflammatory effect of 6-MP.

Discussion

These investigations indicate that a profound antiphlogistic action can be added to the growing list of properties of 6-mercaptopurine. The effect on the inflammatory cycle was apparent when low doses were given for extended periods, but was evident in a few days when larger amounts were administered. When 6-MP treatment was discontinued, the inflammatory response reverted to normal within a day or two. The inhibition of inflammation was not correlated with development of leukopenia or neutropenia, or with weight loss, all suggested as explanations for some of the effects of 6-MP. Achievement of the anti-inflammatory effect in an adrenalectomized animal provided an indication that the antiphlogistic action of the drug was not mediated by adrenal stimulation.

Earlier studies in our laboratory showed that migration of lymphocytes into an area of inflammation depended on the prior presence of neutrophils at the site.[6] In the neutropenic animal the appearance of lymphocytes during the early phases of the inflammatory cycle was delayed. However, the inflammatory sequence returned to normal if such an animal received an injection of viable neutrophils at the site of inflammation. Presumably the neutrophils at the inflammatory site acted to produce substances inducing lymphocytic exudation.

Although normal numbers of neutrophils appeared at the usual time in the altered inflammatory process in 6-MP-treated rabbits, the normal migration of lymphocytes did not occur. Several hypotheses are attractive, and each suggests further experimental approaches. First, it may be that 6-MP affects the neutrophils or the connective tissue so that substances chemotactic for lymphocytes are not produced at the inflammatory site. If this is the case, mononuclear cells may be normally responsive and yet not be induced to participate in the inflammatory process. A second possibility is that lymphocytes and monocytes, although not destroyed by 6-MP, are injured by this treatment and rendered unresponsive to the usual chemotactic substances or processes active at the site of inflammation. A third hypothesis is that 6-MP injures or destroys the circulating mononuclear cells which are active in acute inflammation, assumed to be a small fraction of the total mononuclear cell population. Hence, the inflammatory process would be drastically altered,

without significant reduction in the total numbers of circulating mononuclear cells.

The demonstrated lag in the anti-inflammatory effect of 6-MP, particularly with small doses, must also be considered in relation to these hypotheses. If only a small selective portion of the total population of lymphocytes participates in the inflammatory reaction, and 6-MP blocks their production to a greater or less degree depending on dose, a variable period of time would elapse before the cell population falls below the critical level and the inflammatory response is affected. Studies with tritiated thymidine labeling of lymphocytes have shown that normally a small percentage of the lymphocyte population is capable of dividing.[10] Studies are under way to determine whether these cells are also the mononuclear cells that participate in inflammation.

Summary

Six-mercaptopurine treatment markedly altered the inflammatory cycle in rabbits, virtually eliminating the participation of hematogenous mononuclear cells in this process. The anti-inflammatory effect of 6-MP developed only after a period of treatment with the drug, and the duration of this period was inversely related to dose. Preliminary observations indicated that the same antiphlogistic effect was obtained in the absence of the adrenal glands.

Hypotheses regarding the mechanism of this action of 6-MP have been considered.

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[*Illustrations follow*]

Legends for Figures

Specimens were stained with a combination of Wright and Giemsa stains.

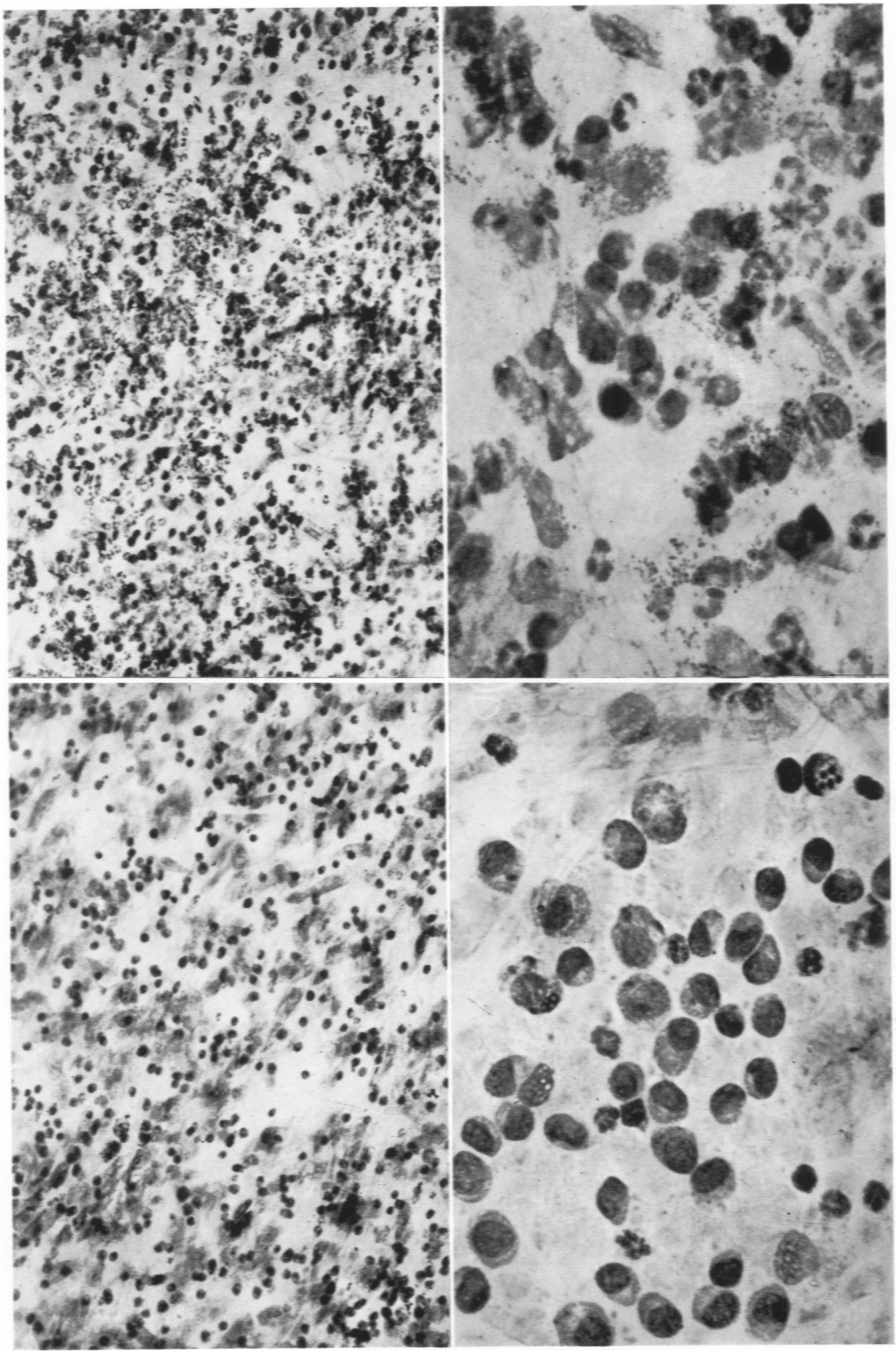
Fig. 1. Connective tissue spreads, showing the cellular response to an inflammatory stimulus in a normal rabbit.

A. In a 4-hour specimen neutrophils and lymphocytes appear in an area adjacent to a blood vessel. × 100.

B. In a higher power view the neutrophils and small lymphocytes are more clearly shown. × 430.

C. In a 48-hour specimen a dense mononuclear cell infiltration is manifest. × 100.

D. A higher power view exhibits mononuclear cells in all stages of development from small lymphocytes to active macrophages. × 430.



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1C 1D

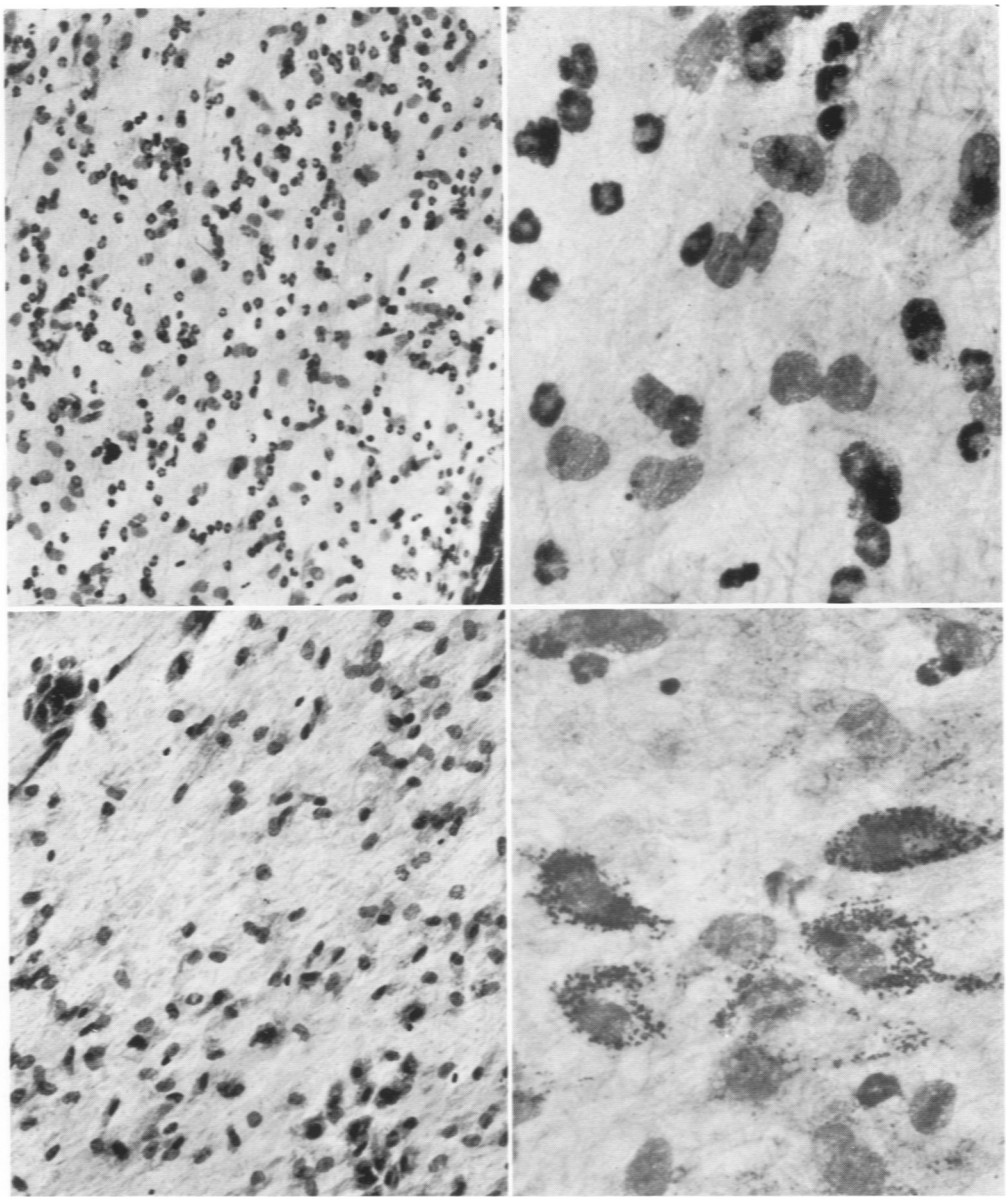

FIG. 2. Connective tissue spreads, showing the cellular response to an inflammatory stimulus in a rabbit pretreated with 12 mg. per kg.of 6-mercaptopurine for 14 days.

A. An 8-hour specimen exhibits neutrophils but no lymphocytes in an area adjacent to a blood vessel. × 100.

B. At higher power the neutrophils are readily identified. × 430.

C. In a 48-hour specimen tissue histiocytes contain carbon particles. No blood cells are present. × 100.

D. In a higher power view the tissue macrophages are seen to contain injected carbon. No hematogenous lymphocytes or round cells are present. × 430.