THE EFFECT OF AZATHIOPRINE (IMURAN®) ON THE CELL CYCLE OF PROMONOCYTES AND THE PRODUCTION OF MONOCYTES IN THE BONE MARROW*

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Azathioprine (Imuran®) has been shown to cause a reduction in the number of monocytes in the peripheral blood, the rate and extent of the reduction being related to the dose of the drug and the duration of its administration. A high dose of azathioprine—daily 200 mg/kg, which is the maximum tolerated dose in mice—administered over a period of 9 days, causes an almost complete reduction of the circulating monocytes; this effect is reversible, because when treatment is stopped the number of monocytes start to increase 24–48 h later.[1] Daily administration of a low dose (3 mg/kg), which is about equivalent to a nontoxic, immunosuppressive, anti-inflammatory dose in man, results after 9 days in a reduction of about 50% in the number of monocytes.[1] The number of peritoneal macrophages, however, only decreases when a high dose of the drug is given over a longer period; a low dose has virtually no effect.[1]

Azathioprine also suppresses the acute inflammatory reaction in that the numbers of monocytes in the peripheral blood and macrophages at the site of inflammation show virtually none of the increase that would normally occur in the absence of the drug. The extent of this anti-inflammatory action is also dependent on the dose of azathioprine administered.[1]

Labeling studies with [^{3}H]thymidine indicated that azathioprine inhibits the formation of monocytes both during the normal steady state and in an acute inflammatory response. Because azathioprine is an antimetabolite interfering with DNA synthesis (1, 2), it can be expected to inhibit the mitotic activity of the direct precursor of the monocyte in the bone marrow, the promonocyte. Since the kinetic parameters of the promonocytes in normal mice are known in great detail, this problem could be approached directly (3, 4).

The present report describes the effect of azathioprine on the mitotic activity of the promonocytes and the quantitative aspects of monocyte production during azathioprine treatment.

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[1] Gassmann, A. E., and R. van Furth. 1975. The effect of azathioprine (Imuran®) on the kinetics of monocytes and macrophages during the normal steady state and acute inflammatory reaction. *Blood J. Hematol.* In press.

Materials and Methods

Animals. This investigation was done in specific pathogen-free male Swiss mice (25–30 g) obtained from the Central Institute for the Breeding of Laboratory Animals, TNO, Bilthoven, The Netherlands.

Azathioprine and 6-Mercaptopurine. Azathioprine (Imuran, 50-mg vials, freeze dried) was kindly provided by Burroughs Wellcome & Co., London, England. Immediately before use, 5 ml pyrogen-free distilled water was added to the vial. When necessary, further dilutions were made such that the vol to be injected was always 0.5 ml. The drug was administered subcutaneously in the nuchal region at intervals of 24 h unless otherwise stated. The doses applied were 200, 100, 50, or 3 mg/kg body weight.

6-mercaptopurine (Burroughs Wellcome & Co.) was used only for in vitro experiments. A fresh stock solution (3,000 μg/ml) was prepared daily by dissolving 6-mercaptopurine in 0.1 ml 1 N NaOH and 0.9 ml medium 199 (Microbiological Associates Inc., Bethesda, Md.). The stock solution was further diluted with medium 199 before addition to the culture medium.

Bone Marrow Cultures. The methods used to obtain bone marrow samples, prepare cell suspensions, and culture these cells, have been described in detail elsewhere (3–5). Cell suspensions made from the bone marrow of individual femurs were processed separately. The culture medium consisted of medium 199, 20% newborn calf serum (NBCS)[2] (Grand Island Biological Co., Grand Island, N. Y.), 200 U/ml penicillin G, and 50 μg/ml streptomycin. The cell suspensions were first incubated in a Leighton tube for 2 h to allow the cells to attach to the cover slip. After the supernate had been removed and the cell layer on the cover slip washed three times by vigorous shaking in 1.5 ml medium 199, the attached cells were incubated again with 2 ml culture medium for an additional 4 h. After a total incubation time of 6 h, the cells on the cover slip were washed again three times, quickly air dried, and fixed for 30 min in absolute methyl alcohol.

Bone Marrow Cell Counts. The total number of nucleated cells per femur was counted in the bone marrow cell suspension (diluted 1:20 with Türk's solution containing 6% acetic acid) in a Bürker hemocytometer. The total number of monocytes per femur was calculated from the total number of nucleated cells in the bone marrow cell suspension and the percentage of monocytes which was obtained from differential counts of 200 nucleated bone marrow cells in Giemsa-stained preparations made with a sedimentation apparatus (6). The total number of promonocytes per femur was computed with the promonocyte-to-monocyte ratio determined in 6-h bone marrow culture preparations and the total number of bone marrow monocytes.

For each mouse, cell counts were done in both femurs; the values given per femur represent the means of four mice. Since in mice the bone marrow of two femurs accounts for 11.8% of the total bone marrow mass (7), the total number of promonocytes and monocytes per mouse could be calculated.

Peripheral Blood Monocytes. Monocytes of the peripheral blood were studied in tail-vein blood as described in an earlier paper (4). The blood vol of mice weighing 25 g is about 3 ml (8), and the total number of peripheral blood monocytes per mouse was calculated on this basis.

Labeling with [^3H]Thymidine. In vitro labeling of mononuclear phagocytes was performed by incubating the bone marrow cells for 6 h in medium containing 0.1 μCi/ml [^{3}H]thymidine (sp act 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.). To avoid underestimation of the mitotic activity, the medium used for harvesting the bone marrow also contained [^{3}H]thymidine. The cultures were processed and prepared for autoradiography as described elsewhere (3).

In vivo labeling was done by one intravenous injection of 1 μCi/g body weight [^{3}H]thymidine into a tail vein. The bone marrow samples harvested at the chosen time points were cultured for 6 h in a nonradioactive culture medium and prepared for autoradiography as already described (3).

The labeling index at each time point represents the mean value of four mice obtained in two femurs. The total numbers of labeled promonocytes and monocytes were calculated with the values of the labeling index and the total number of cells per mouse.

Autoradiography. Autoradiography and staining of the developed slides were as described elsewhere (5).

Cytophotometric Measurement of Nuclear DNA of Promonocytes and Bone Marrow Monocytes. Bone marrow cell suspensions were cultured for 6 h, washed, quickly air dried, and fixed with absolute methanol for 15 min at room temperature, followed by postfixation in 4% formaldehyde

[2] *Abbreviations used in this paper:* AU, arbitrary units; NBCS, newborn calf serum.

solution for 2 h. After the cells on the cover slip had been stained according to a Feulgen technique for quantitative purposes, the DNA content of the nuclei was determined cytophotometrically with an integrating microdensitometer of the Deeley type (Barr and Stroud, Glasgow, England) at 5,600 A, using an oil-immersion fluorite objective (40×, numerical aperture 0.75) as described by James (9). On each slide the nuclei of 20 granulocytes, 20 monocytes, and 40 promonocytes were measured.

As a reference for the diploid value, we used the Feulgen DNA content of the granulocytes on the same slide. The mean values of the Feulgen-DNA content of granulocytes and monocytes were similar (SD less than 5%). With the mean value obtained for the monocytes, the Feulgen-DNA content of the promonocyte nuclei was calculated for each slide and expressed in arbitrary units (AU). The mean diploid value was taken, via a correction factor, as 100 AU; all readings were multipled by the same correction factor. Cells with a Feulgen-DNA content of 100 AU ± 2 SD are considered diploid, and cells with tetraploid values of Fuelgen-DNA will lie within 200 AU ± 2 SD.

Calculation of Monocyte Production. The total production of monocytes was calculated as described previously (4) with the equation:

$$P_{0\rightarrow t} = (I_{bm} \times N_{bm})_t + (I_{bl} \times N_{bl})_t - (I_{bl} \times N_{bl})_{t-1} + k\int_0^t (I_{bl} \times N_{bl})_t \, dt,$$

in which $P_{0\rightarrow t}$ = total number of labeled monocytes produced in time period 0 to t, I = labeling index, N_{bm} = total number of bone marrow monocytes, N_{bl} = total number of blood monocytes, t = time, k = exponential disappearance rate constant of monocytes from the peripheral blood, and $\int_0^t (I_{bl} \times N_{bl})_t$ = area under the curve of the numbers of labeled blood monocytes from $t = 0$ to $t = t$. In the azathioprine experiments k values were taken as determined in similar experiments without drug treatment (4): for mice treated with azathioprine alone $k = 0.03974\ h^{-1}$, and for azathioprine-treated mice with an acute inflammation $k = 0.06946\ h^{-1}$ for the first 24 h and thereafter $k = 0.03974\ h^{-1}$.

Identification of the Cells. The criteria used for the morphological characterization of the promonocytes, bone marrow monocytes, and peripheral blood monocytes have been described elsewhere (3, 5).

Results

The Effect of Azathioprine on the Number of Promonocytes and Bone Marrow Monocytes. Since the number of monocytes in the peripheral blood decreases during treatment with azathioprine,[1] it was of interest to study the effect of this drug on the number of monocytes and promonocytes in the bone marrow compartment.

Treatment of mice for 4 days with 200 mg/kg azathioprine showed that the number of promonocytes increased during the first 48 h from 3.86×10^4 to 5.86×10^4 cells/femur, and then decreased to 2.38×10^4 at 96 h, or 24 h after the last azathioprine injection (Fig. 1). When the animals were treated with 3 mg/kg azathioprine for a period of 9 days, the number of promonocytes remained constant (Fig. 1).

The number of bone marrow monocytes in mice treated with 200 mg/kg azathioprine decreased from 23.76×10^4 to 5.95×10^4 monocytes/femur at 96 h, which is 37.5% of the normal value. After azathioprine administration was stopped, the number of monocytes increased gradually (Fig. 1). When the animals were treated longer than 4 days with this high dose of azathioprine the number of monocytes dropped to 0.2×10^4/femur at 216 h. The numbers of nucleated cells in these samples were too low for reliable differential counts and therefore the monocyte-to-promonocyte ratio in the 6-h bone marrow culture could not be determined. During the treatment with 3 mg/kg azathioprine the number of bone marrow monocytes decreased gradually, reaching its lowest value (i.e., 10.73×10^4 monocytes/femur) at 216 h (Fig. 1).

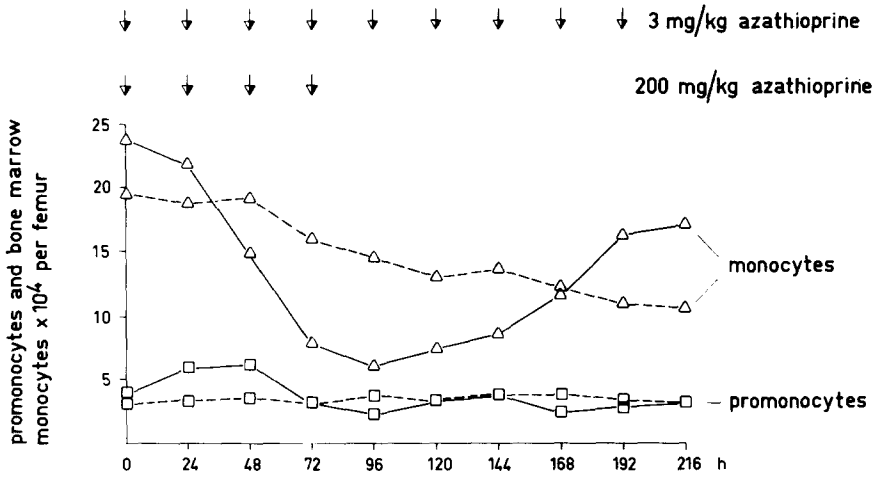

FIG. 1. The effect of treatment with 200 mg/kg (—) or 3 mg/kg (----) azathioprine on the numbers of promonocytes and bone marrow monocytes.

The Effect of Azathioprine on the In Vitro and In Vivo [^{3}H]Thymidine Labeling of Promonocytes. Since it is known that azathioprine inhibits cellular proliferation and causes a decrease in the number of both peripheral blood and bone marrow monocytes, azathioprine would be expected to inhibit the mitotic activity of the direct precursor of the monocytes, the promonocyte. To investigate this point, the effect of this drug on the percentage of promonocytes synthesizing DNA was determined.

First the effect of azathioprine added in vitro to bone marrow cultures from normal mice incubated in the presence of 0.1 μCi/ml [^{3}H]thymidine was studied. Since in vivo azathioprine is converted into 6-mercaptopurine, i.e. the active antimetabolic compound, similar cultures were prepared with this drug. The effect of both drugs on the labeling index of peritoneal macrophages was also determined. The results show that neither azathioprine nor 6-mercaptopurine added in vitro affected the labeling index of the promonocytes and monocytes in 6-h bone marrow cultures (Table I). Also, there was no decrease in the labeling index of peritoneal macrophages cultured for 2 h except in the cultures with 30 μg/ml azathioprine (Table I); longer incubation (24 h) made no difference.

Next, bone marrow samples from mice treated for 1–4 days with 200 mg/kg azathioprine were incubated for 6 h in the presence of 0.1 μCi/ml [^{3}H]thymidine. In normal animals the in vitro labeling index of the promonocytes amounted to 50.9%. During azathioprine treatment, significantly ($P < 0.001$) higher labeling indices (between 93.7 and 92.3%) were found from 72 to 120 h; when treatment was stopped, these indices decreased (Fig. 2 *a*).

In animals treated for 9 days with a low dose of azathioprine (3 mg/kg) the in vitro labeling indices also increased significantly ($P < 0.001$), reaching 84.1 and 90.0% after the 5th day of treatment (Fig. 2 *b*). To verify this increase of the in vitro labeling index, a similar experiment was performed in which cell labeling was done in vivo. Mice treated with the high dose (200 mg/kg) of azathioprine

received 1 μCi/g [^{3}H]thymidine intravenously 1 h before they were killed. The results demonstrate that the in vivo labeling indices of the promonocytes were also significantly ($P < 0.001$) increased, the highest value being 92.0% at 96 h (Fig. 2 *c*).

These results are contrary to expectation, since no decrease due to inhibition of DNA synthesis occurred in the promonocyte-labeling index during in vivo treatment with azathioprine. Because azathioprine does not lower the number of promonocytes in the bone marrow either, these results mean that the total

TABLE I

The Effect of Azathioprine and 6-Mercaptopurine on the In Vitro Labeling of Promonocytes, Bone Marrow Monocytes, and Peritoneal Macrophages

Concentration of drug in medium	Azathioprine			6-mercaptopurine		
	Labeled promonocytes*	Labeled bone marrow monocytes*	Labeled peritoneal macrophages‡	Labeled promonocytes*	Labeled bone marrow monocytes*	Labeled peritoneal macrophages‡
µg/ml	%	%	%	%	%	%
0	50.3	0.4	3.0	50.8	0.5	2.8
0.3	51.3	0.8	3.8	49.8	0.6	3.0
3.0	54.4	0.3	4.9	50.6	0.5	3.0
30.0	50.3	0.1	1.3	49.6	0.4	2.1

* Cultured for 6 h in medium with 0.1 μCi/ml [^{3}H]thymidine.
‡ Cultured for 2 h in medium with 0.1 μCi/ml [^{3}H]thymidine.

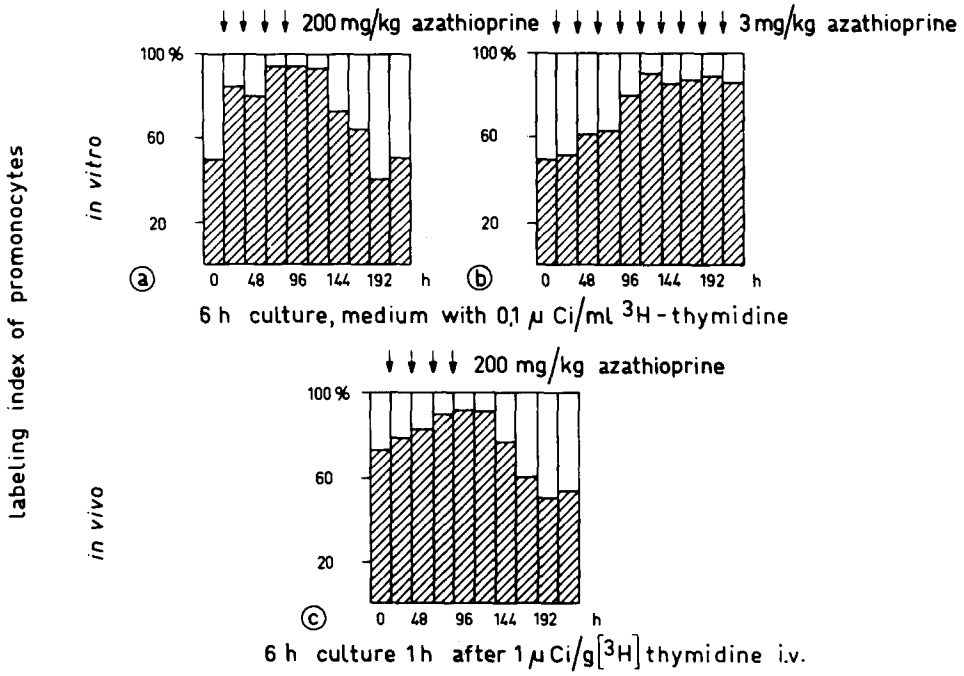

FIG. 2. The effect of treatment with 200 mg/kg or 3 mg/kg azathioprine on the labeling index of promonocytes after 6 h of incubation with [^{3}H]thymidine (*a* and *b*), and 1 h after an intravenous injection with [^{3}H]thymidine (*c*).

number of promonocytes synthesizing DNA is not reduced by azathioprine treatment. Since the production of monocytes is inhibited, it is likely that this drug affects the cell cycle of promonocytes at another and later stage. It was therefore relevant to determine the DNA synthesis time and cell cycle time of these cells.

The Effect of Azathioprine on the DNA Synthesis Time and Cell Cycle Time of the Promonocytes. The method used to determine the DNA synthesis time from the percentages of labeled promonocytes after three intravenous injections of 1 μCi/g [^{3}H]thymidine given precisely 1 h apart, has been described previously (4) and the results pertaining to normal mice were taken from that publication.

It was again clear that during treatment with azathioprine the labeling index 1 h after the first [^{3}H]thymidine injection (i) was higher than in normal mice (Table II). The hourly increment of the labeling index (Δi) was reduced during treatment with azathioprine.

TABLE II

*DNA-Synthesis Time (t_s) and Cell-Cycle Time (t_c) in the Normal Steady State and During Treatment with Azathioprine**

	Normal steady state‡	Acute inflammation‡		Azathioprine (200 mg/kg)		Azathioprine (3 mg/kg)		Azathioprine (3 mg/kg; 96 hr) + acute inflammation (12) §
		12 h§	24 h§	48 h‖	96 h‖	96 h‖	192 h‖	
i	72.9%	69.8%	76.7%	83.6%	91.6%	81.7%	81.4%	81.5%
Δi	6.2%	9.2%	6.0%	1.0%	0.4%	4.4%	4.8%	5.1%
t_s	11.8 h	7.6 h	12.8 h	83.6 h	229.0 h	18.6 h	17.0 h	16.0 h
t_c	16.2 h	10.8 h	16.7 h	(100.0 h)	(250.0 h)	22.8 h	20.9 h	19.6 h

i, initial labeling index of promonocytes 1 h after first injection of [^{3}H]thymidine; Δi, mean hourly increment of promonocyte-labeling index after second and third injections of [^{3}H]thymidine; t_s, i/Δi; and t_c, $t_s \times 100/i$.

* Mice labeled with 1, 2, or 3 intravenous injections of 1 μCi/g [^{3}H]thymidine.

§ Time after intraperitoneal injection of 1 ml NBCS.

‖ Duration of azathioprine.

‡ From reference 4.

The DNA-synthesis time of the promonocytes (t_s) was computed by dividing the initial labeling index by the increment of the labeling index per hour (i/Δi) (10). In mice treated with 3 mg/kg azathioprine the DNA synthesis time was significantly ($P < 0.001$) prolonged, on the average by 6 h, and mice treated with 200 mg/kg azathioprine had an extremely long DNA synthesis time (Table II).

The cell cycle time of the promonocyte (t_c) was computed by dividing the DNA-synthesis time by the initial labeling index ($t_s \times 100/i$). During treatment with 3 mg/kg azathioprine the cell cycle time was on the average 5.7 h longer than in normal mice (Table II). Application of this calculation to the data from the animals treated with the high dose of azathioprine would give extremely long generation times, which are meaningless. The results of these experiments show unequivocally that during treatment with azathioprine the DNA sysnthesis time

of the promonocytes is prolonged, thus making the cell cycle time significantly ($P < 0.001$) longer.

Cytophotometric Determination of the DNA Content of Promonocytes and Bone Marrow Monocytes. Since azathioprine causes a prolongation of the DNA synthesis time and thus retards the mitotic activity of the promonocytes it was of interest to find out whether the bone marrow of these animals had an increased number of promonocytes with a tetraploid DNA content.

Cytophotometric determination of the Feulgen-DNA content of nuclei of bone marrow monocytes and promonocytes was done in normal mice and in mice treated for 96 h with 200 mg/kg azathioprine. Compared with the diploid standard of the granulocytes in the same preparation, the nuclei of the monocytes in all of the samples had a diploid DNA content. In normal mice 15% of the promonocytes showed a diploid content of Feulgen-DNA, 42.5% lay higher than diploid and lower than tetraploid, and 42.5% were tetraploid (Fig. 3).

The azathioprine-treated mice showed a gradual decrease in the percentage of diploid promonocytes (at 96 h none of the promonocytes had a diploid

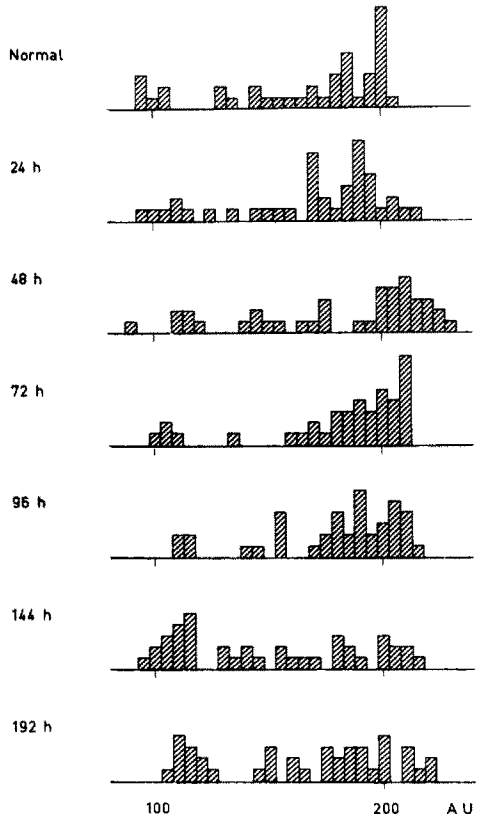

FIG. 3. The effect of treatment with 200 mg/kg azathioprine (given up to 96 h) on the Feulgen-DNA content of the promonocytes, expressed in AU. At 48, 72, and 96 h the percentage of tetraploid promonocytes (about 200 AU Feulgen-DNA) was significantly increased ($P < 0.01$). After azathioprine treatment was stopped, the percentage of diploid-promonocytes (about 100 AU) increased.

Feulgen-DNA content). The percentage of promonocytes with a tetraploid or higher Feulgen-DNA value gradually increased to 60% at 72 h. The samples taken at 192 h, when azathioprine treatment had been stopped for 96 h, showed 12.5% of the promonocytes to have a diploid and 32.5% a tetraploid Feulgen-DNA value (Fig. 3). Statistical analysis (rank order test) showed that the increase in the Feulgen-DNA content of the promonocytes between 48 and 96 h was significant ($P < 0.001$). The results of this cytophotometric study thus confirm the conclusion that during azathioprine treatment the promonocytes synthesize DNA, and indicate that after DNA synthesis the cells do not proceed via the G_2-phase to mitotic division.

In Vivo Labeling with [^3H]Thymidine During Azathioprine Treatment. Labeling of promonocytes and monocytes was studied in mice treated for 4 days with 200 mg azathioprine and given a single intravenous injection of 1 μCi/g [^{3}H]thymidine 1 h before the first dose of azathioprine. The labeling index of the promonocytes increased from 75% at 1 h to 80% at 24 h after [^{3}H]thymidine administration and then decreased, which is in agreement with the results of in vitro [^{3}H]thymidine labeling (Fig. 2 *a*) and those obtained in the labeling experiment performed to determine the DNA synthesis time (Table II). The total number of labeled promonocytes per mouse, computed with the labeling indices and total number of cells (calculated with data from Fig. 1), increased during the first 24 h and then leveled off, which is quite different from the course found in normal animals (Fig. 4). The increase in the total number of labeled bone marrow and peripheral blood monocytes in these animals, calculated with the labeling indices and total number of cells per mouse (calculated with data from Fig. 1 and footnote 1), was considerably lower than the increase in normal animals (4) (Fig. 4), indicating a decreased monocyte production.

The Effect of Azathioprine on Monocyte Production. With the data of the previous experiment (Fig. 4) the monocyte production during treatment with 200 mg/kg azathioprine is calculated to be 65.3×10^4 cells during the first 24 h; after that, monocyte production is virtually arrested.

In the next experiment monocyte production was studied after 4 days of

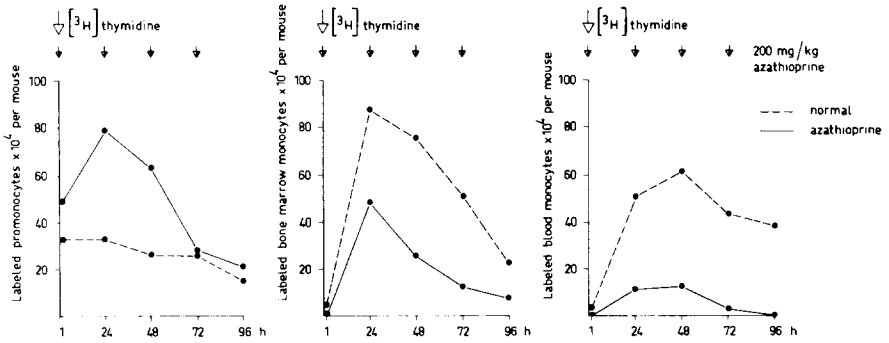

FIG. 4. The effect of treatment with 200 mg/kg azathioprine (—) on the total number of labeled promonocytes, bone marrow monocytes, and peripheral blood monocytes, when 1 μCi/g [^{3}H]thymidine was given intravenenously 1 h before the first dose of azathioprine. The data for the normal mice (----) are taken from reference 4.

treatment with 200 mg/kg azathioprine. In these mice, which were given an intravenous injection of 1 μCi/g [^{3}H]thymidine 1 h after the last dose of azathioprine, the total number of labeled monocytes in the bone marrow and peripheral blood (Fig. 5) was considerably lower than that found in normal mice (4). The total production of labeled monocytes calculated with these data amounted to 10.0×10^4 cells during the first 24 h and continued to increase (Fig. 5), but remained more than 90% lower than in normal mice (5).

Since treatment with a high dose of azathioprine considerably reduced the production of monocytes, the effect of a low dose was studied. Mice were treated daily with 3 mg/kg azathioprine and after 4 days received an intravenous injection of 1 μCi/g [^{3}H]thymidine. The monocyte production (calculated from the labeling indices and total number of bone marrow and peripheral blood monocytes) amounted to 1.18×10^6 cells during the first 24 h after labeling, which is 75% of the production in normal mice (Fig. 6). After that, monocyte production did not increase any further, in contrast to normal mice.

The Effect of Azathioprine on Monocyte Production during an Acute Inflammatory Reaction. Since treatment with 3 mg/kg azathioprine reduced the number of mononuclear phagocytes in an inflammmatory exudate[1] and also diminished the monocytosis[1] usually occurring during such an inflammatory response (4, 5) we investigated monocyte production under these conditions. Mice undergoing the same treatment as in the preceding experiment were given an intraperitoneal injection of NBCS 1 h after the [^{3}H]thymidine injection. In these animals the calculated monocyte production amounted to 1.17×10^6 cells during the first 24 h and thereafter increased slightly (Fig. 6). At 96 h the difference between this monocyte production and that of azathioprine-treated

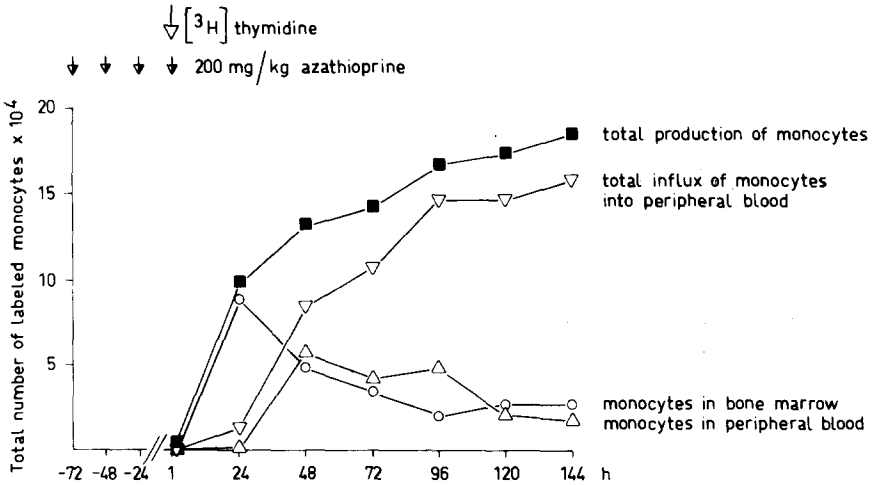

FIG. 5. Total production of labeled monocytes after treatment with 200 mg/kg azathioprine. A single intravenous injection of 1 μCi/g [^{3}H]-thymidine was given 1 h after the last dose of azathioprine. The influx of monocytes into the peripheral blood is the sum of the labeled peripheral blood monocytes and the number of labeled monocytes that have left the circulation. The total production of labeled monocytes is the sum of the number of labeled bone marrow monocytes and the total influx of monocytes into the peripheral blood.

mice without an inflammatory reaction was not significant but the production was much lower than that found during an inflammatory reaction in untreated mice (Fig. 6).

To obtain more information about the effect of azathioprine on monocyte production during an acute inflammation, the phases of the cell cycle of the promonocytes were determined, as described above, 12 h after an acute peritoneal inflammation had been provoked with NBCS in mice treated for 4 days with 3 mg/kg azathioprine. Here, the DNA synthesis time amounted to 16.0 h and the cell cycle time to 19.6 h (Table II). Both of these values are very similar to those obtained in mice given only 3 mg/kg azathioprine, but are significantly

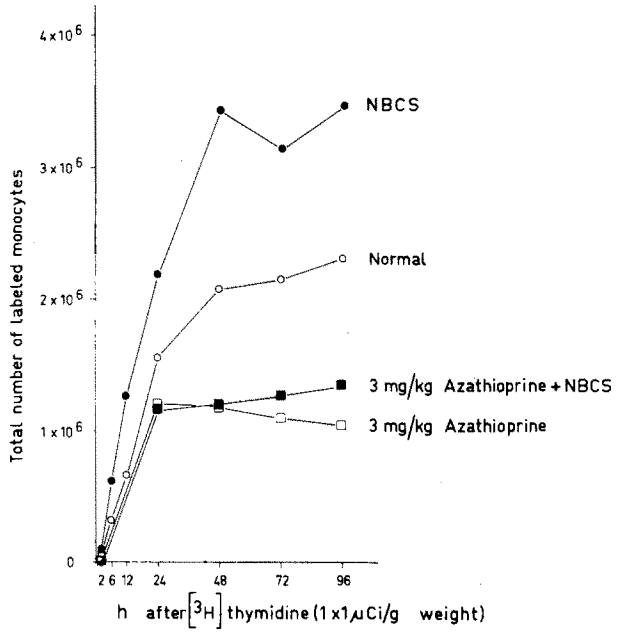

FIG. 6. Total production of labeled monocytes in mice treated daily with 3 mg/kg azathioprine, with and without an acute inflammatory reaction provoked with an intraperitoneal injection of 1 ml NBCS given 1 h after the [^{3}H]thymidine. The single injection of 1 µCi/g [^{3}H]thymidine was given after 4 days of pretreatment with azathioprine. For comparison, the findings in the absence of azathioprine (from reference 4) are given.

($P < 0.001$) longer than those in normal mice with a provoked inflammatory reaction (Table II). With the number of promonocytes (4.56×10^5 cells/mouse) found at the 12th h of the inflammatory response in azathioprine-treated mice and the cell cycle time of the promonocytes, the monocyte production was calculated (4) to be 0.47×10^6 monocytes/h (Table III).

Discussion

The main conclusion to be drawn from this study is that azathioprine reduces the formation of monocytes in the bone marrow because this drug strongly

TABLE III
The Effect of Azathioprine on the Rate of Monocyte Production

Conditions	Period	Monocyte production	
		Calculation A*	Calculation B‡
		$\times 10^5/h$	$\times 10^5/h$
Normal		0.65	0.62
Acute Inflammation§	0–12	1.06	
	12–24	0.78	
	at 12		0.96
	at 24		0.70
High dose azathioprine‖	0–24	0.27	
	72–96	0.04	
Low dose azathioprine¶	72–96	0.49	
	at 96		0.47
Low dose azathioprine** and acute inflammation**	0–24	0.49	
	at 12		0.47

R, production rate; $P_{0\to t}$, total number of labeled monocytes produced in time period 0 to t; N_{pro}, total number of promonocytes; t, time; and t_c, cell cycle time.

* $R = P_{0\to t}/t$.

‡ $R = 2 \times N_{pro}/t_c$.

§ Intraperitoneal injection of 1 ml NBCS at 0 h.

‖ 200 mg/kg azathioprine sc daily.

¶ 3 mg/kg azathioprine sc daily.

** Intraperitoneal injection of 1 ml NBCS after 4-day pretreatment with azathioprine.

inhibits the mitotic activity of the promonocytes. The mechanism underlying this effect on the promonocytes is as follows: although these cells still synthesize DNA, this phase of the cell cycle is prolonged, the cell cycle of the promonocytes is arrested late in the DNA synthesis phase or in the postsynthesis phase, called the G_2 phase, and the next phase, mitosis, does not occur.

What observations support this conclusion? In animals treated with azathioprine the number of monocytes in the peripheral blood[1] and bone marrow decreases. However, the effect of azathioprine is reversible, since the number of monocytes in the bone marrow and peripheral blood rises again after administration of the drug has been stopped. The in vivo labeling studies show that the formation of labeled monocytes is reduced in dependence on the duration of the administration of the drug: 24 h after a high dose of azathioprine the production is still 50% of that in normal mice, but after 4 days this value has dropped to 10%. A reduced monocyte production could be expected to result from a diminished mitotic activity of the promonocytes and a significantly prolonged cell cycle time was indeed found in azathioprine-treated animals. In the low dose treatment this prolongation was about 5.5 h; in the high dose treatment extremely long generation times were estimated.

It is of interest that the intervention of this drug in the cell cycle of the

promonocytes could be localized. In azathioprine-treated animals the number of promonocytes increased initially, which is compatible with a prolonged cell cycle time of these cells. Furthermore, in the in vivo and in vitro labeling experiments with [^{3}H]thymidine the labeling indices were significantly higher than those in normal mice. This was unexpected, because if this drug inhibits DNA synthesis (1, 2), lower values should be found. Determination of the DNA synthesis time demonstrated that the DNA synthesis phase of the cell cycle was prolonged in azathioprine-treated mice with and without an acute inflammation. Cytophotometric analysis next demonstrated a significantly higher percentage of promonocytes with a tetraploid DNA content. All of these findings lead to the conclusion that during azathioprine treatment the promonocytes pass through the DNA synthesis phase of the cell cycle but are then arrested near the end of the DNA synthesis phase or in the G_2 phase.

These in vivo observations on the time of the effect of azathioprine in the cell cycle of the promonocytes seem to be novel. This drug and 6-mercaptopurine also reduce the numbers of other categories of leukocytes (e.g., granulocytes and lymphocytes) (references 11–16 and footnote 1) but no detailed analysis of how this effect occurs in vivo has been published as yet. For the mixed lymphocyte reaction it has been reported that 10 μg/ml azathioprine inhibits the incorporation of [^{3}H]thymidine in vitro, whereas a 10 times higher concentration is cytotoxic (17). However, the authors mention a stimulation of the incorporation of this label when very low concentrations (0.1 and 0.001 μg/ml) were used. In the present study the addition of azathioprine to bone marrow cultures did not affect DNA synthesis. Another communication describes the inhibition of in vitro DNA synthesis of mouse spleen cells by azathioprine, but does not throw light on the mode of action of this drug on the cells (18). In vivo administration of azathioprine was also found to inhibit DNA synthesis by spleen, kidney, duodenum, thymus, lymph node, and normal liver cells in rats (19). Of interest there is the observation that the administration of azathioprine suppresses the stimulation of DNA synthesis in liver cells after partial hepatectomy, but the regenerating liver of these rats showed a population of liver cells apparently in the G_2 phase that were stimulated to divide (19). This finding again indicates that azathioprine blocks the cell cycle just before or in the G_2 phase, as was found in the present study, but liver cells in the G_2 phase can apparently proceed to the mitosis phase. A similar effect on the cell cycle was observed in a transplantable carcinoma in rabbits (20), epithelial cells of the small intestine of mice (21), and cultures of Chinese hamster cells treated with bleomycin (22, 23); in Ehrlich tumor cells in mice (24, 25), mouse fibroblast cultures (25), HeLa cells (26) and Chinese hamster cells (26) treated with nitrogen mustard; in cultured human leukocytes (27), and Ehrlich ascites tumor cells in mice (28) treated with rubidomycin (daunomycin); and in fibroblast cultures exposed to melphalan (29), mitomycin C (29), and myeleran (30).

The rate of monocyte production during azathioprine treatment can be computed according to two methods (A and B) (Table III), as had been done previously for the normal steady state and an acute inflammatory response (4). Comparison shows that after 24 h of treatment with a high dose of azathioprine the rate of monocyte production is about 40% and after 96 h is less than 10% of the

rate in the normal steady state. Treatment with a low dose of azathioprine only gives a reduction of 20%, but when an acute inflammation is provoked the rate of monocyte production does not increase as in animals not treated with azathioprine (Table III). The calculations (B) based on the total number of promonocytes and the promonocyte cell cycle time during treatment with 3 mg/kg azathioprine agree very well with the rate of monocyte production derived from the completely independent experimental data on total monocyte production (Table III). This agreement once again confirms our earlier conclusion (3, 4) that the division of one promonocyte gives rise to two monocytes, since calculation B is based on this premise.

Insight into the kinetics of the mononuclear phagocytes during azathioprine treatment can be obtained by comparing the data on the total production of labeled monocytes, the influx of monocytes into and their efflux from the peripheral blood compartment, and the migration of labeled monocytes into the peritoneal cavity (taken from footnote 1), with corresponding data obtained during the normal steady state and acute inflammatory reaction (4) (Fig. 7). From this comparison it is evident that treatment with 3 mg/kg azathioprine considerably reduces monocyte production, and that fewer labeled monocytes enter and leave the circulation. During an inflammatory response in these azathioprine-treated animals, monocyte production is not augmented but the passage of these cells through the circulation is slightly accelerated. The number of labeled monocytes produced and transported through the circulation, is, however, considerably lower than during an acute inflammatory response in animals without azathioprine.

Not only do fewer cells leave the circulation, but in addition, only 10% of these monocytes arrive in the inflammatory exudate in the peritoneal cavity of azathioprine-treated animals; in animals without azathioprine and with an inflammatory reaction, 40% of the cells leaving the circulation migrate to the site of inflammation. This might indicate that azathioprine not only affects mono-

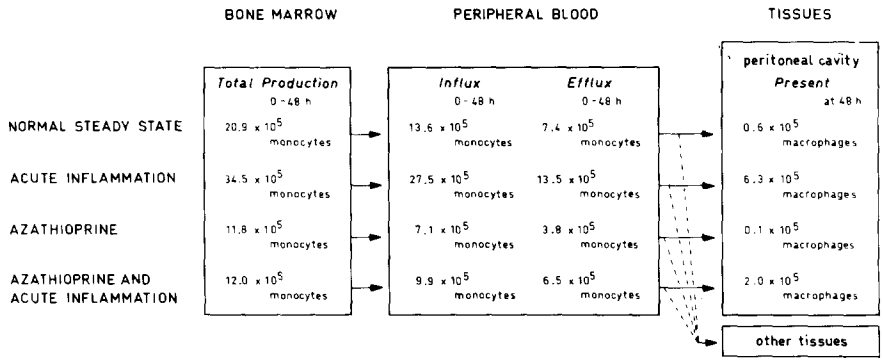

FIG. 7. The total production of labeled monocytes in the bone marrow, their transit through the peripheral blood compartment, and their migration into the peritoneal cavity during treatment with a low dose (3 mg/kg) of azathioprine and during an acute inflammation in azathioprine-treated mice. For comparison, the course during the normal steady state and an acute inflammation (data from reference 4) is indicated.

cyte production but also affects the formation of a cellular exudate at the site of the inflammation in a different way.

The effect of azathioprine on monocyte production is quite different from the effect of another category of drug, namely glucocorticosteroids, which also affect the inflammatory reaction. The latter only inhibits promonocyte division and monocyte production slightly (about 20%), but cause an impaired release of these cells from the bone marrow into the circulation (31). Glucocorticosteroids also cause monocytopenia in the peripheral blood, most probably by a sequestration of these cells in a still unknown pool (32). During an acute inflammatory response in glucocorticosteroid-treated animals there is little augmentation (20%) of the production of monocytes (unpublished observation), there is no monocytosis caused by recruitment of cells from the bone marrow, and the influx of monocytes into the site of lesion is markedly (90%) reduced (32). The difference between the mechanisms underlying the action of glucocorticosteroids (31, 32) and azathioprine may lead to an additive effect of these drugs, which are widely used in combination because of their anti-inflammatory and immunosuppressive actions.

Summary

The present communication concerns the effect of azathioprine on the mitotic activity of promonocytes and the production of monocytes. In vitro and in vivo labeling with [^{3}H]thymidine showed that during azathioprine treatment the promonocytes synthesize DNA and that, contrary to expectation, the labeling index increases. Cytospectrophotometric determination of the Feulgen-DNA content of the promonocytes during azathioprine treatment showed an increase in the percentage of tetraploid promonocytes, and determination of the various phases of the cell cycle showed significantly increased DNA synthesis and cell cycle times as compared with the normal steady state.

On the basis of these results it can be concluded that azathioprine arrests the cell cycle of the promonocytes late in the DNA synthesis phase or in the postsynthesis (G_2) phase and mitosis does not occur. This timing of the effect of azathioprine had not been previously observed.

The diminished mitotic activity of the promonocytes during azathioprine treatment depressed monocyte production. During treatment with 3 mg/kg azathioprine the cell cycle time of the promonocytes was on the average 5.5 h longer than in the normal steady state and the rate of monocyte production was reduced by 70%.

During an acute inflammatory reaction too, monocyte production is affected by azathioprine. In animals not treated with azathioprine but with an acute inflammation the cell cycle time becomes shorter and the monocyte production increases, but animals treated with (3 mg/kg) azathioprine do not show this effect.

The kinetics of the monocyte also changes under the low dosage of azathioprine. As consequence of the diminished production of monocytes, far fewer

(about 50%) monocytes enter and leave the circulation than during the normal steady state. During an acute inflammatory reaction the numbers in transit through the circulation are slightly augmented but remain considerably lower than in nonazathioprine-treated animals. As a result, the inflammatory exudate contains 70% less macrophages than that of animals not treated with azathioprine.

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