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A cyclic GMP- and G-kinase-dependent effect of azathioprine on migration by human neutrophils

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Abstract. Relatively high concentrations of azathioprine had an inhibitory effect on interleukin 8 (IL-8)- or formyl-methionyl-leucyl-phenylalanine-activated (fMLP)-chemotaxis by human neutrophils. However, application of low concentrations of azathioprine in a concentration gradient gave a chemotactic stimulation to random migration. Stimulation of migration was maximal at a concentration of 5 μM azathioprine; at higher concentrations stimulation decreased again. The activating effect of azathioprine is located in the mercaptopurine moiety of the molecule, since mercaptopurine also stimulated neutrophil migration. In contrast to some other chemotactic agents such as fMLP and IL-8, an activating concentration (5 μM) of azathioprine did not cause an upregulation of CD11b expression on neutrophils in suspension. High concentrations of azathioprine (1 mM) inhibited CD11b expression of fMLP- or IL-8- activated neutrophils; the latter could explain the inhibitory effect of azathioprine. Azathioprine caused a transient stimulation of cGMP level; inhibitors of guanylate cyclase inhibited azathioprine-stimulated migration, suggesting that cGMP was associated with the stimulating effect of azathioprine on migration. Antagonists of cGMP-dependent protein kinase (G-kinase) strongly inhibited azathioprine-activated migration, indicating that the effect of azathioprine proceeds via G-kinase. The antagonists had only a marginal effect on inhibition of IL-8-activated chemotaxis by high concentrations of azathioprine, thus the G-kinase seems not to be of great importance on the inhibitory effect of azathioprine.
Key words. Neutrophil; azathioprine; mercaptopurine; migration; chemotaxis; cGMP; G-kinase; CD11b.

Azathioprine is a drug with immunosuppressive and anti-inflammatory properties. It is widely used in immunologically mediated diseases and in transplantation. Because of its anti-inflammatory effect it is used in the treatment of rheumatic disorders, although the drug has a number of side effects [1–4]. Together with drugs as penicillamine, gold salts, sulphasalazine, hydroxychloroquine and methotrexate, it is classified as a disease-modifying antirheumatic drug. These drugs alter the course of the disease by retarding its underlying progression, but they do not necessarily cure the disease. They are characterized by a delayed onset of clinical action beyond that of the non-steroidal anti-inflammatory drugs [5].

Neutrophils are suspected to play a role in inflammatory conditions because of their ability to migrate to the inflamed site, and to release inflammation-promoting substances. For that reason the effect of azathioprine on neutrophil functions has been tested by some investigators. Either no effect [6, 7] or an inhibitory effect [8, 9] on neutrophil chemotaxis was found.

A number of sulfur-containing agents are known to affect neutrophil migration [10–12]. This could not be demonstrated when the effect of the agents on migration induced by an optimal concentration of a chemoattractant was tested, but was clearly demonstrated when the effect on random migration was measured in the absence of other chemoattractants. In this study we considered the stimulating and inhibitory effect of azathioprine on neutrophil migration, and made an attempt to characterize the nature of the modulating effect, especially with regard to the role of cGMP and the role of sulfhydryl groups.

Materials and methods

Isolation of human neutrophils. Neutrophils were isolated from the buffy coat of blood of healthy donors. The buffy coat was diluted with a four-fold volume of heparinized medium, and layered on top of Ficoll-amidotrizoate (d = 1.077). After centrifugation the pellet was resuspended, and starch was added to sediment erythrocytes. After sedimentation the neutrophil-containing supernatant was collected and centrifuged. The remaining erythrocytes were removed by hypotonic hemolysis, and the neutrophils suspended in medium. The cells consisted of more than 95% of neutrophils, and were more than 99% viable, as determined by Trypan blue exclusion. The medium used consisted of 140 mM NaCl, 5 mM KCl, 10 mM glucose, 0.5% bovine serum albumin and 20 mM Hepes pH 7.3.

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Unless otherwise stated the medium was supplemented with 1 mM Ca^{2+} and 1 mM Mg^{2+} during the experiments. The final cell suspension during the experiments contained 3×10^6 neutrophils per ml.

Electroporation of neutrophils. Neutrophils were electroporated according to the method of Grinstein and Furuya [13], with minor modifications. The electropermeabilization procedure was carried out at room temperature. When permeabilization was carried out at 0 °C the cells were not able to migrate. Neutrophils (3×10^6 per ml) in permeabilization medium (135 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM Hepes pH 7.0, 10 mM glucose and 0.5% bovine serum albumin, BSA), were placed in the cuvette of a BioRad Gene Pulser. The cells were exposed to two discharges of 14.75 kV/cm from a 25 μF capacitor. Between the two discharges the cell suspension was stirred with a plastic pipette. After permeabilization and mixing, 0.2 ml of the suspension was placed in the upper compartment of the Boyden chamber. To compare electroporated neutrophils with control neutrophils the latter cells were also suspended in permeabilization buffer.

Neutrophil migration. Cell migration was measured with the Boyden chamber technique, as described by Boyden [14] and modified by Zigmond and Hirsch [15]. The two compartments of the chamber were separated by a cellulose acetate Millipore filter (type SSWP, thickness 150 μm) with a pore size of 3 μm. Medium supplemented with 1 mM Ca^{2+}, 1 mM Mg^{2+}, and 0.5% BSA was present in both the upper and lower compartment, unless otherwise indicated. Neutrophils were placed in the upper compartment of the chamber, followed by incubation for 40 min at 37 °C. After migration the filters were fixed and stained and the distance travelled in micrometers into the filter was determined according to the leading front technique [15]. Chemotactic assays were carried out in duplicate and the migration distance of the neutrophils was determined at five different filter sites.

Cyclic GMP assay. Neutrophils (final concentration 2×10^7 cells per ml) were exposed to reagents at 37 °C for the indicated time. Subsequently 1 ml 3.5% perchloric acid was added, and the resulting mixture was stored overnight in the freezer. The solution was neutralized by adding 0.5 ml saturated (22 °C) $NaHCO_3$. After 10 min the mixture was centrifuged for 3 min at 2000 rpm. To 100 μl of the supernatant 50 μl of radioactive cGMP and 50 μl antibody from the radio-immunoassay kit (Amersham, England) were added. After mixing the solution was kept on ice for 90 min, after which 1 ml icecold 60% $(NH_4)_2SO_4$ was added. The solution was mixed, and kept on ice for a further 10 min, and centrifuged. The supernatant was carefully removed, and the residue taken up in 1.1 ml water. 1 ml of the solution was mixed with 4 ml scintillation fluid (299, Packard), and counted in the scintillation counter. Known amounts of cGMP were treated in the same way as the cells, and were used for the calibration curve.

Flow cytometric analysis of CD11b expression. Neutrophils (3×10^6 per ml) were incubated for the indicated lengths of time with the desired compounds at 37 °C. After stimulation a sample of 67 μl cell suspension was mixed with 25 μl monoclonal antibody (FITC-anti-CD11b, diluted 1:16) and 8 μl buffer, and incubated for 30 min on ice in the dark. After dilution with 4 ml medium, the mixture was centrifuged, and the pellet suspended in 0.5 ml paraformaldehyde (1% in 0.9% NaCl). The suspension was mixed, and placed in the dark at 4 °C for 30 min. Subsequently the suspension was centrifuged and washed two times with medium, after which the cells were resuspended in 0.5 ml medium. From this suspension 10,000 cells were analysed with a flow cytometer (FACScan, Beckton Dickinson) within 24 h. The values given are the mean fluorescence intensity, which correlates directly with CD11b antigen density. The values given were corrected for autofluorescence and non-antigen specific antibody binding by measuring cells which were not treated with antibody, and cells which were treated with mouse IgG.

Statistical analysis. All mean values for the chemotactic assays are arithmetical means ± S.E. of four experiments. In those cases where random migration or activated migration was considerably different for different cell batches, values were expressed as percentage of control. Significances were calculated with Student's t-test; a value of $p < 0.05$ was considered as statistically significant.

Materials. Azathioprine, formyl-methionyl-leucyl-phenylalanine (fMLP), and methylene blue were purchased from Sigma Chemical Co. IL-8 was obtained from R & D Systems Europe, Abingdon, England. The compound LY-83583 (6-anilino-5,8-quinolinedione) was obtained from Calbiochem, Bierges, Belgium. The G-kinase antagonists R_p-pCPT-cGMPS (R_p-8-(4-chlorophenylthio-guanosine-3-prime-, 5′-cyclic monophosphorothioate) R_p-Br-cGMPS (R_p-8-bromoguanosine-3′,5′-cyclic monophosphorothioate), R_p-Br-PET-cGMPS (R_p-8-bromo-β-phenyl-1,N^2-ethenoguanosine-3′,5′-cyclic monophosphorothioate), were from Biolog, Bremen, Germany. The other chemicals were obtained from Sigma Chemical Co., Saint Louis, Missouri, and were of the highest purity available.

Results

Inhibition of migration by azathioprine. Chemotactic migration induced by IL-8 or by fMLP was inhibited when neutrophils were pretreated with azathioprine at relatively high (>40 μM) concentrations (fig. 1). The length of time of preincubation with azathioprine had only a limited influence on the degree of inhibition. When cells were not preincubated for 30 min, as shown

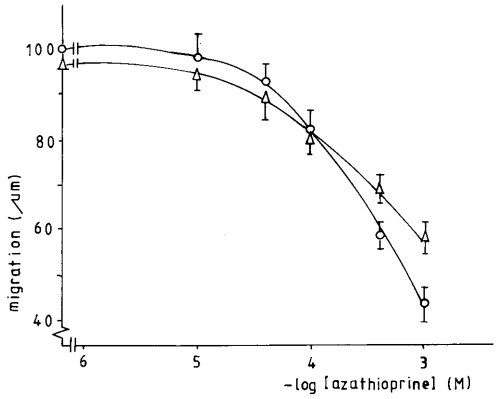

Figure 1. Inhibition of fMLP- or IL-8-activated chemotaxis by azathioprine. Cells were preincubated with the indicated concentrations of azathioprine for 30 min, and subsequently placed in the upper compartment of the Boyden chamber. The chemotactic peptide fMLP (—△—) (10^{-9} M) or 4×10^{-9} M IL-8 (—○—) was present in the lower compartment of the Boyden chamber. Inhibition was significant for concentrations of 100 μM azathioprine or higher.

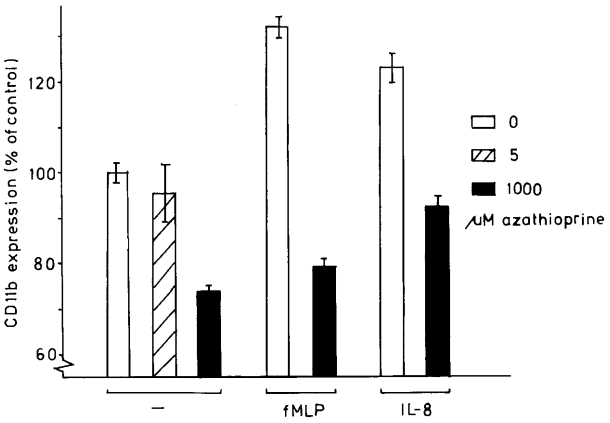

Figure 3. The effect of azathioprine on CD11b expression of resting neutrophils, and of fMLP- or IL-8-activated neutrophils. Cells were incubated with the stimulating agents for 10 min, after which CD11b expression was measured as described in Methods. The concentration of fMLP was 10^{-9} M, that of IL-8 was 4×10^{-9} M.

in figure 1, but added without preincubation to the cells immediately before the chemotaxis experiment, the migration of IL-8-activated cells was 100.1 ± 1.9 μm without azathioprine, and 46.2 ± 1.7 μm with 1000 μM azathioprine. Without preincubation, migration of fMLP-activated neutrophils was 96.8 ± 1.8 μm in the absence of azathioprine, and 59.2 ± 2.0 μm in the presence of 1000 μM azathioprine.

Activation of migration by azathioprine. Depending on the concentration, azathioprine caused a stimulatory or inhibitory effect on neutrophil migration when it was present in the lower compartment of the Boyden chamber. The stimulatory effect increased up to a concentration of 5 μM azathioprine, and decreased again at higher concentrations. Azathioprine concentrations of 100 μM or more slightly inhibited random migration (fig. 2). The stimulating effect of azathioprine was most pronounced when it was present in the lower compartment of the Boyden chamber. Only a slight stimulation was found when the drug was present in both compartments with the cells (fig. 2).

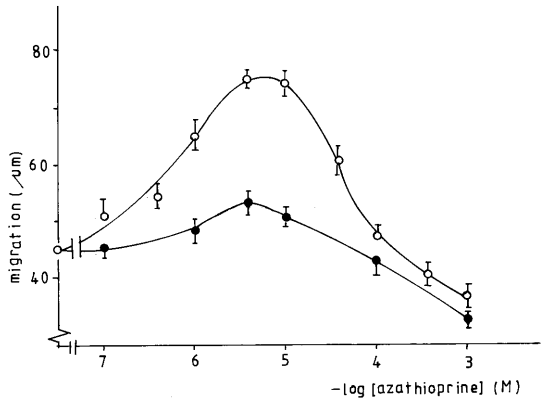

Figure 2. The effect of increasing concentrations of azathioprine on neutrophil migration. The indicated concentration of azathioprine was placed in the lower compartment only (—○—), or was present in both compartments (—●—).

Chemotaxis induced by an optimal concentration of either fMLP or IL-8 was no further enhanced by 5 μM azathioprine, present in the lower compartment of the Boyden chamber. Migration in the absence of azathioprine was 89.2 ± 1.9 μm for fMLP; with fMLP + 5 μM azathioprine the migration was 88.5 ± 1.7 μm. For IL-8 the migration was 95.3 ± 1.6 μm in the absence of azathioprine, and 95.4 ± 1.5 in its presence.

CD11b upregulation. In contrast to a chemotactic concentration of fMLP, azathioprine in a chemotactic concentration (10 μM) did not cause an enhancement of CD11b adhesion molecules on the cell surface of neutrophils in suspension (fig. 3). Pretreatment of neutrophils with an inhibitory concentration of azathioprine (1 mM) caused a strong reduction in CD11b expression of neutrophils exposed to a chemotactic concentration of fMLP or of IL-8 (fig. 3).

Role of cGMP. Exposure of neutrophils to azathioprine (5 μM) caused a transient increase in cGMP level of neutrophils. The effect of azathioprine on cGMP level was time dependent. The effect was most pronounced after incubation for one minute. At longer incubation times cGMP level rapidly declined (fig. 4). Pretreatment of neutrophils with two inhibitors of cGMP accumulation, methylene blue or LY 83583 [16–18], resulted in a strong reduction of azathioprine-induced enhancement of migration (fig. 5). Inhibition of IL-8-activated chemotaxis by 1 mM azathioprine was not reversed by methylene blue or by LY83583 (table 1); the interpretation of these results is somewhat hampered by the fact that methylene blue and especially LY83583 inhibited IL-8-activated migration by themselves. For the study of antagonists of G-kinase we used electroporated neutrophils, because this allows the application of a given concentration to the cell interior, and a direct compari-

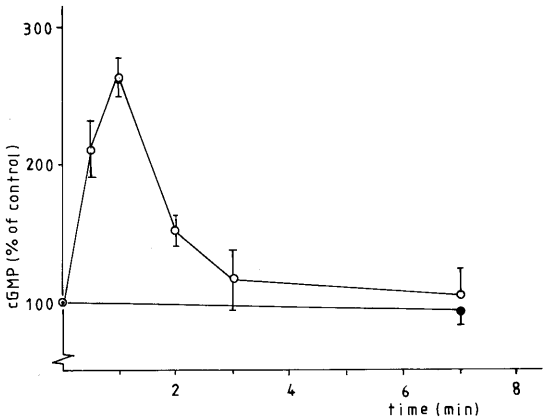

Figure 4. Time course of the effect of 5 μM azathioprine on cGMP level of neutrophils, as compared with control cells. (—●—): control cells; (—○—): azathioprine-treated cells. Values are expressed as a percentage of the cGMP content of control cells at t = 0 (100% = 6.72 pmoles cGMP per mg protein).

son with the effect of cGMP on migration. Pretreatment of neutrophils with three antagonists of G-kinase [19, 20], R_p-pCPT-cGMPS, R_p-Br-PET-cGMPS and R_p-Br-cGMPS, strongly inhibited azathioprine-stimulated migration by electroporated cells (fig. 6). There was a strong resemblance with the effect of cGMP on migration: cGMP caused a stimulation of neutrophil migration [21], which was strongly inhibited by the G-kinase antagonists. The three antagonists had little effect on chemotactic migration activated by IL-8, and had only a marginal effect on inhibition of IL-8-activated migration by 1 mM azathioprine (table 1). Under the conditions of our experiments the antagonists had a moderate effect on chemotactic migration activated by fMLP (results not shown).

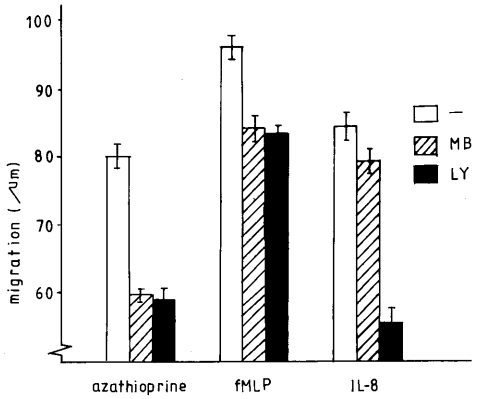

Figure 5. Inhibition of activated neutrophil migration by pretreatment with inhibitors of cGMP accumulation. Cells were preincubated without reagents (—), with 20 μM methylene blue (MB), or with 5 μM LY 83583 for 30 min at 37 °C. Subsequently the cells were placed in the upper compartment of the Boyden chamber. In the lower compartemt 5 μM azathioprine, 10^{-9} M fMLP or 4×10^{-9} M IL-8 was present. Although the effect of methylene blue on IL-8-activated migration was less than in the other cases, the difference between untreated cells and methylene blue treated cells was nevertheless statistically significant ($p < 0.05$).

Table 1. The effect of inhibitors of guanylate cyclase and G-kinase antagonists on azathioprine-induced inhibition of IL-8-activated chemotaxis.

	IL-8 activated migration (μm)	
	—	+1 mM azathioprine
—	100.0 ± 2.7	56.8 ± 1.9
LY83583	68.1 ± 1.9	51.1 ± 2.4
Methylene blue	93.4 ± 2.1	40.6 ± 1.7
R_p-pCPT-cGMPS	95.8 ± 1.9	58.2 ± 1.8
R_p-Br-PET-cGMPS	95.2 ± 1.7	61.3 ± 2.0
R_p-Br-cGMPS	95.0 ± 2.0	60.8 ± 1.8

Cells were preincubated with or without 1 mM azathioprine, in the absence of reagents (—), in the presence of 20 μM methylene blue, 5 μM LY83583, 4 nM R_p-pCPT-cGMPS, 20 nM R_p-Br-PET-cGMPS, or 100 nM R_p-Br-cGMPS for 30 min at 37 °C, and subsequently placed in the upper compartment of the Boyden chamber. IL-8 (4×10^{-9} M) was present in the lower compartment of the Boyden chamber. Values are expressed as a percentage of IL-8-activated migration in the absence of other agents.

Role of sulfhydryl groups. In the presence of the reducing sulfhydryl compound dithiothreitol, random migration and fMLP-activated migration were only slightly affected (table 2). Azathioprine-activated migration, as well as IL-8 activated chemotaxis, were strongly inhibited by dithiothreitol (table 2).

To evaluate the role of the surrounding of the sulfur group in azathioprine, and the role of surface sulfhydryl groups on the neutrophil membrane, the cells were pretreated with the hydrophilic sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), which cannot readily permeate the plasma membrane. Azathioprine is a thioether derivative of mercaptopurine. It appeared that mercaptopurine was effective in stimulating neutrophil migration (table 3). The activating effect of azathioprine and of IL-8 was only moderately affected by DTNB pretreatment, in contrast with the effect of fMLP (table 3).

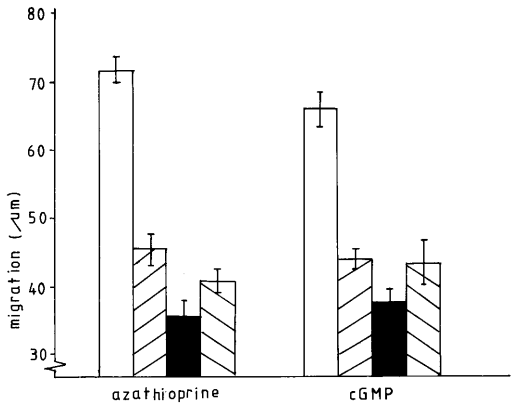

Figure 6. The effect of antagonists of G-kinase on azathioprine- and cGMP-activated migration by electroporated neutrophils. □: control; ▨: 4 nM R_p-pCPT-cGMPS; ▩: 20 nM R_p-Br-PET-cGMPS; ▧: 100 nM R_p-Br-cGMPS. Cells were preincubated with the antagonists for 15 min at 37 °C, and then placed in the upper compartment of the Boyden chamber. Azathioprine (5 μM) was present in the lower compartment of the Boyden chamber; cGMP (5 μM) was present in both compartments.

Table 2. The effect of dithiothreitol on azathioprine-, IL-8- and fMLP-activated migration.

	Migration (μm)	
	—	+0.1 mM dithiothreitol
—	46.6 ± 1.9	44.2 ± 1.6
Azathioprine	79.7 ± 1.7	47.3 ± 1.5
fMLP	98.8 ± 1.8	94.7 ± 2.0
IL-8	99.1 ± 2.2	63.1 ± 2.1

Dithiothreitol was present in both compartments of the Boyden chamber; azathioprine (5×10^{-6} M) and fMLP (10^{-9} M) were present in the lower compartment only.

Table 3. The effect on migration of pretreatment of neutrophils with DTNB.

	Migration (μm)	
	Control cells	DTNB-treated cells
—	51.6 ± 1.8	46.0 ± 2.7
Azathioprine	81.7 ± 2.3	72.8 ± 1.8
Mercaptopurine	84.1 ± 1.9	75.9 ± 2.1
fMLP	93.8 ± 1.9	60.3 ± 1.5
IL-8	84.1 ± 2.0	79.3 ± 1.7

Cells were pretreated with or without 200 μM DTNB for 30 min at 37 °C. Subsequently the cells were centrifuged, resuspended in fresh medium, and placed in the upper compartment of the Boyden chamber. Azathioprine (5 μM), mercaptopurine (5 μM), fMLP (10^{-9} M) and IL-8 (4×10^{-9} M) were present in the lower compartment of the Boyden chamber.

Discussion

The results show that azathioprine is able to inhibit or potentiate neutrophil migration, depending on the concentration and the method of application. The stimulating effect of azathioprine is predominantly chemotactic. This may be concluded from the observation that stimulation of migration is much higher when azathioprine is in the lower compartment only, than when it is present in both compartments of the Boyden chamber. Under conditions where the stimulatory effect of azathioprine would normally be chemokinetic, the presence of azathioprine in the lower compartment would result in only a small increase in migration as compared with the situation where azathioprine was present in both compartments. Because the reverse is true it may be concluded that the effect of azathioprine is chemotactic rather than chemokinetic.

In accordance with the results of studies by other investigators, the stimulatory effect is not observed when the cells are pretreated with azathioprine, and the effect on migration induced by other activators was studied. Moderate concentrations had no effect, as found previously [5, 6]. We could not reproduce the inhibition by extremely low concentrations of azathioprine, found by Di Stefano et al. [8]. We found inhibition of chemotaxis by azathioprine, but only at concentrations higher than 40 μM. It seems unlikely that this inhibitory effect of azathioprine could explain its beneficial effect because the azathioprine concentrations in vivo are lower, unless it is accumulated at specific places. The activating effect of azathioprine on neutrophil migration occurs at physiologically relevant concentrations. Given the fact that the neutrophil is suspected of contributing to inflammation, the activating effect does not explain the beneficial action of azathioprine in inflammatory conditions.

We have previously found that a variety of sulfur-containing agents (glutathione and a number of antirheumatic agents) had chemotactic properties for neutrophils, and that all these agents caused an increase of cGMP level in neutrophils [22]. An increase in cGMP level has often been associated with an increased migration [22–27]. Azathioprine fits in this pattern. The question arises whether the increase in cGMP level is the cause of enhanced migration, or that it is a consequence of a reaction which is associated with enhanced migration. There are a few observations which are compatible with the view that an enhancement of cGMP production is the cause of enhanced migration. We have demonstrated that the application of cGMP (up to a certain concentration) to the cell interior of electroporated neutrophils causes an enhanced migration of these cells [21]. Nitric oxide (NO), an agent which is a well-known activator of soluble guanylate cyclase, also causes an increase of migration [28]. When the cGMP-enhancing effect of azathioprine is counteracted with agents like methylene blue and LY83583, the effect on migration is greatly reduced. Although there are several indications which suggest that cGMP is involved in azathioprine-activated migration, there is a peculiar difference between the effect of azathioprine (and a number of other cGMP-enhancing compounds) on one hand, and the effect of cGMP on electroporated cells on the other. Azathioprine-activated migration is predominantly chemotactic, while migration of electroporated neutrophils by cGMP is chemokinetic. We have no satisfactory explanation for this phenomenon.

The effect of cGMP in the signal transduction pathway may be exerted in a number of ways: ion channels, cGMP-modulated phosphodiesterases, and cGMP-dependent kinases (G-kinases). It appears that the effect of azathioprine occurs via a G-kinase, because migration can be completely annulled by low concentrations of a number of specific G-kinase antagonists. This also applies to some other systems which we have studied, such as NO-activated migration [28], and activation of (electroporated) cells by cGMP itself [21]. The role of cGMP in azathioprine-modulated migration does not rule out the possibility that another agent is an intermediate between azathioprine and the eventual effect on migration. Azathioprine could cause the release of such an agent, which than would act as a chemotactic agent, rather than azathioprine itself. This situation has been found, for example, for methotrexate, an agent which

causes release of adenosine, which can modulate neutrophil migration [29, 30].

Azathioprine is a thioether. Conversion of the compound into the corresponding thiol (mercaptopurine) does not diminish the stimulating effect, because mercaptopurine also stimulates neutrophil migration. This implies that the activating effect of azathioprine is confined to the mercaptopurine moiety of the molecule. The reducing sulfhydryl compound dithiothreitol strongly inhibits the activating effect of azathioprine, with little effect on agents such as fMLP. The effect of dithiothreitol is not due to conversion of azathioprine into mercaptopurine, because the latter is also an migration-activating compound. It seems conceivable that intact disulfide bridges on the cell surface are required for the stimulating effect of azathioprine. Cellular ecto-sulfhydryl compounds might be involved in the effect of sulfur compounds, because they can act as intermediates in sulfide-disulfide interactions. The inactivation of ecto-sulfhydryl groups has little effect on azathioprine and IL-8-activated migration, suggesting that sulfide-disulfide interactions on the cell surface do not play a role, in contrast with some other stimulating sulfur-containing compounds such as glutathione, where DTNB pretreatment completely eliminates the activating effect of glutathione [10]. DTNB has a significant effect on fMLP-activated migration. Comparable findings were done by Goetzl and Hoe [31], who discovered that treatment of neutrophils with the impermeant covalent sulfhydryl reagent chloromercuribenzene sulfonate result in inhibition of fMLP-activated migration. It could mean that intact sulfhydryls on or near the fMLP receptor are essential for activated migration.

In vivo migration by neutrophils is regulated by adhesion molecules on the cell membrane. CD11b plays a particular role in migration in vivo. Some activators of migration, such as fMLP, cause an upregulation of CD11b expression on the cell surface. The relation between CD11b upregulation and the extent of migration is somewhat obscure, because adhesion is required for migration, but migration is impaired when adhesion is either too strong or too weak. The determination of an exact correlation between expression of adhesion molecules and migration in vitro is hampered by differences in the experimental conditions during measurement: CD11b upregulation is measured for cells in suspension, while migration is measured for adherent cells. Under the conditions of our experiments there is little correlation between upregulation of CD11b in suspension, and migration of neutrophils, activated by either azathioprine or fMLP. Low (chemotactic) concentrations of azathioprine had little effect on CD11b expression of resting cells, while the highest concentration of azathioprine abolished the upregulation of CD11b expression by fMLP or IL-8. It seems that upregulation of CD11b is therefore not required for stimulated migration by azathioprine. However, it is possible that the inhibition of fMLP- or IL-8-activated migration by high concentrations of azathioprine is somehow associated with the inhibition of fMLP-or IL-8-activated CD11b expression.

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