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STIMULATION OF RABBIT POLYMORPHONUCLEAR LEUKOCYTE LOCOMOTION BY D-PENICILLAMINE

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Abstract—Treatment of polymorphonuclear leukocytes (PMNs) with penicillamine has little effect on chemotaxis of the cells towards fMet-Leu-Phe. In the absence of fMet-Leu-Phe however, penicillamine has a strong effect on PMN locomotion. Penicillamine-induced enhancement of PMN migration is for an important part due to a chemokinetic effect, but there is also a chemotactic effect. Pretreatment of PMNs with membrane-impermeable sulfhydryl reagents has little effect on random locomotion, but completely abolishes the activating effect of penicillamine. This suggests that for the activating effect of penicillamine intact sulfhydryl groups on the outer surface of the PMN are required. Extracellular calcium is no absolute requirement for PMN migration, neither for random locomotion nor for the activating effect of penicillamine. Removal of intracellular Ca^{2+} with membrane-penetrating Ca^{2+}-complexing agents strongly inhibit penicillamine-induced enhancement of PMN migration, underlining a role for intracellular calcium. Penicillamine causes an increase of cGMP level in PMNs. Though a simple relationship seems unlikely it is possible that the enhancement of PMN migration, and the increase of cGMP level by penicillamine are related processes.

The polymorphonuclear leukocyte (PMN) plays not only an important role in the defense of the body against foreign invaders, but may also cause inflammation due to its ability to release proteolytic enzymes and toxic oxygen metabolites. Substances which interfere with these processes, or with the movement of PMNs to the site of inflammation may also interfere with the defence function or with inflammation.

D-Penicillamine is a widely-used drug in the treatment of rheumatoid arthritis, though it has a number of side effects [1, 2]. It interferes with a number of PMN functions [3–5], and this might be relevant for an understanding of its beneficial effect *in vivo*. The drug has little effect on chemotaxis of PMNs towards chemotactic agents [6, 7], though it has been observed that it could restore decreased chemotaxis in patients responding to several months of D-penicillamine treatment [8]. Depending on the experimental procedure an inhibition of chemotaxis towards chemotactic agents could be induced by D-penicillamine, especially when the cells were preincubated with the drug for a long time [9]. On the other hand, sometimes a significant enhancement was found [3].

While some functions of the PMN are inhibited by penicillamine there are others which are stimulated. Oben and Foreman [10] found that lysosomal enzyme release, and phagocytosis of bacteria are significantly enhanced by the drug. In the same study a similar effect was found for glutathione.

Recently we found that glutathione has only a slight effect on PMN chemotaxis towards chemotactic peptide, but had a strong chemokinetic effect on its own, together with a small but significant chemotactic effect. The similarity between the effects of glutathione and penicillamine reported by Oben and Foreman [10] prompted us to study the effects of penicillamine on PMN locomotion in the absence of other chemotactic agents. It appeared that penicillamine had a strong chemokinetic effect and a moderate chemotactic effect on rabbit PMNs. The expression of these effects however, strongly depended on the experimental conditions.

MATERIALS AND METHODS

Isolation of polymorphonuclear leukocytes. PMNs were isolated from the peritoneal cavity of the rabbit [11, 12]. Four hours after intraperitoneal injection with 200 mL isotonic saline containing 1.5 mg/mL glycogen, the exudate was collected by flushing the peritoneal cavity with isotonic saline containing citrate (0.4%, pH 7.4). The cells were centrifuged and washed with medium. The medium used consisted of 140 mM NaCl, 5 mM KCl, 10 mM glucose and 20 mM Hepes, pH 7.3. In addition 1 mM Ca^{2+}, 1 mM Mg^{2+} and 0.5% bovine serum albumin (BSA) were added to the medium before incubation (but after preincubation, if there was one). The final cell suspension during the experiments contained 3×10^6 PMNs per mL.

Chemotaxis. Cell migration was measured with the Boyden chamber technique, as described by Boyden [13], and modified by Zigmond and Hirsch [14]. The two compartments of the chamber were separated by a cellulose acetate Millipore filter 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. Penicillamine was mostly present in the lower compartment only, but in some experiments penicillamine was present in both compartments, as indicated. PMNs were placed in the upper compartment of the chamber, followed

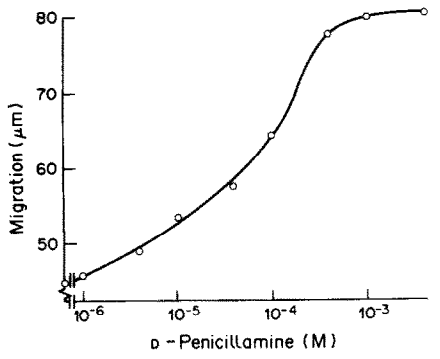

Fig. 1. Effect of increasing concentrations of D-penicillamine in the lower compartment of the Boyden chamber on migration of rabbit PMNs. Values given are the mean of three experiments.

by incubation for 40 min at 37°. 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 [14]. The assays were carried out in triplicate and the migration distance of the PMNs was determined at five different filter sites. The standard deviation was calculated on the basis of all fifteen determinations.

Cyclic GMP assay. PMNs (final concentration 2×10^7 cells per mL) were exposed to reagents at 37° 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°) $NaHCO_3$. 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 radioimmunoassay kit (Amersham, U.K.) were added. After mixing, the solution was kept on ice for 90 min, after which 1 mL ice-cold 60% $(NH_4)_2SO_4$ was added. The solution was mixed, 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. One milliliter 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.

RESULTS

D-Penicillamine caused a strong increase of PMN motility when it was present in the lower compartment of the Boyden chamber. The enhancing effect of penicillamine was concentration-dependent, and occurred in the concentration range between 1 μM and about 1 mM. At concentrations higher than 1 mM no further increase in motility was observed (Fig. 1).

To address the question of how far the enhancement of migration was due to a chemokinetic effect or to chemotaxis, a checkerboard assay [15] was carried out (Table 1). In this assay the results represented on the diagonal represent the chemokinetic component of the enhancement of migration. Though there was a strong chemokinetic effect it appears that the enhanced migration was for an important part due to a chemotactic component (Tables 1 and 2).

The effect of D-penicillamine on migration in the absence of other chemotactic agents was compared with the effect of D-penicillamine on chemotaxis towards the chemotactic peptide fMet-Leu-Phe, under different experimental conditions (Table 2). The enhancing effect of penicillamine in the absence of fMet-Leu-Phe was quite pronounced, and even present when the agent was in the upper compartment only. Migration towards fMet-Leu-Phe however, was only marginally affected when the penicillamine was present in both compartments of the Boyden chamber, or in the lower compartment only. When penicillamine was only present in the upper compartment together with the cells no enhancement of migration was observed. These results however, were obtained with optimal concentrations of fMet-Leu-Phe (10^{-9} M), where the effect of fMet-Leu-Phe is much stronger than that of penicillamine. When sub-optimal concentrations of fMet-Leu-Phe were used migration was enhanced by penicillamine, albeit to a moderate degree. In a separate experiment we found that migration towards 10^{-10} M fMet-Leu-Phe was 78.9 ± 1.3 μm, and that in the presence of 0.5 mM penicillamine (in the lower compartment) the migration towards this fMet-Leu-Phe concentration was 85.4 ± 1.3 μm.

Both forms of penicillamine were effective in enhancing migration. The L-form and the racemic mixture were slightly more effective than the D-form, but the difference was small (Table 3).

The compounds 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *p*-chloromercuribenzene sulfonic acid (PCMBS) are sulfhydryl reagents which have little or no membrane permeating capacity [16, 17]. Pretreatment of PMNs with these agents had little effect on spontaneous locomotion by PMNs. DTNB had no effect at all, and PCMBS had only a slight effect. However, the agents completely inhibited the directed and non-directed increase of locomotion due to penicillamine (Fig. 2). As a comparison the effect of these reagents of fMet-Leu-Phe-stimulated locomotion was considered. While DTNB had only a moderate inhibitory effect, PCMBS completely inhibited the increase of migration due to the presence of fMet-Leu-Phe in the lower compartment of the Boyden chamber (Fig. 2).

Bovine serum albumin was present in most chemotaxis experiments described in the literature, and also in our experiments. This is not only due to the fact that albumin is often a component of the medium, but also because the motility of the cells is enhanced by albumin, thus facilitating the experimental procedure. In order to determine how far the effect of penicillamine was mediated by albumin we compared the effect of penicillamine in the absence and presence of albumin (Table 4). The migration of the cells in the absence of albumin was, as expected, lower than in its presence, but the

Table 1. Migration of neutrophils in different absolute concentrations and concentration gradients of D-penicillamine

Penicillamine concn (mM) in upper compartment	Penicillamine concentrations (mM) in lower compartment				
	0	0.01	0.05	0.1	0.5
0	45.1 ± 0.6				
0.01		50.5 ± 1.1	56.2 ± 1.7 (52)	67.9 ± 1.1 (53)	76.5 ± 1.3 (54)
0.05		56.2 ± 1.8 (55)	55.9 ± 1.5	63.7 ± 1.5 (58)	73.8 ± 1.3 (59)
0.1		55.1 ± 0.9 (61)	59.5 ± 1.3 (62)	63.9 ± 1.1	70.1 ± 1.0 (64)
0.5		60.0 ± 1.3 (62)	61.4 ± 1.0 (64)	62.9 ± 0.8 (65)	65.9 ± 0.9

Values given represent the migration in micrometers into the filter after an incubation time of 40 min, in the presence of the indicated concentrations of D-penicillamine below and above the filter. The values between brackets are the migration values to be expected on the basis of effects on chemokinesis alone (calculated according to Zigmond and Hirsch [14]).

Values given are the means of three experiments (15 determinations) ± SD.

Table 2. Effect of the experimental procedure on the migration-enhancing effect of D-penicillamine

D-Penicillamine (0.5 mM) present in:		Migration (μm)	
Upper compartment	Lower compartment	—	FMLP
−	−	45.0 ± 1.4	106.8 ± 1.5
−	+	79.0 ± 1.1	111.8 ± 2.1
+	+	65.7 ± 0.9	107.1 ± 1.1
+	−	55.4 ± 1.4	105.5 ± 1.1

Migration in the absence of other chemotactic agents (—), or in the presence of 10^{-9} M fMet-Leu-Phe in the lower compartment (FMLP) was established in the presence or absence of 0.5 mM D-penicillamine as indicated.

Values given are the means of three experiments (15 determinations) ± SD.

Table 3. Effect of different stereoisomers of penicillamine on PMN location

	Migration (μm)
—	49.5 ± 1.1
D-Penicillamine	79.8 ± 1.4
L-Penicillamine	84.3 ± 1.9
(±)-Penicillamine	85.1 ± 2.2

Migration was measured in the absence (—) or in the presence of 0.5 mM penicillamine in the lower compartment of the Boyden chamber.

Values given are the means of three experiments (15 determinations) ± SD.

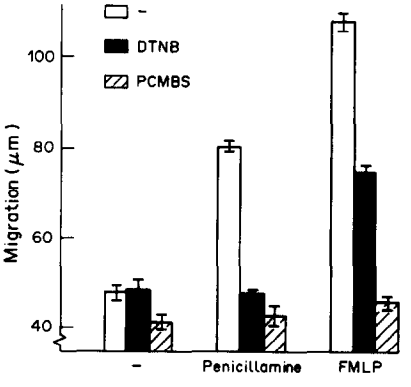

Fig. 2. The effect of pretreatment of PMNs with sulfhydryl reagents on penicillamine or fMet-Leu-Phe activated migration. Cells were pretreated with or without 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) or 0.2 mM *p*-chloromercuribenzene sulfonic acid (PCMBS) for 20 min at 37°. The suspensions were centrifuged, the supernatant was removed, and the cells resuspended in medium, and placed in the upper compartment of the Boyden chamber. In the lower compartment no reagent (—), 0.5 mM D-penicillamine or 10^{-9} M fMet-Leu-Phe (FMLP) was present. Values are the mean of three experiments (15 determinations) ± SD.

percentage increase of migration due to penicillamine was about the same.

Divalent cations have a modulatory effect on PMN chemotaxis. PMNs migrate in the absence of divalent cations, although mostly (dependent on the cell batch) to a somewhat lower degree. The enhancing effect of penicillamine occurred both in the presence and in the absence of divalent cations (Table 4).

Table 4. Effect of albumin and divalent cations on penicillamine-induced enhancement of PMN migration

	Migration (μm) in the presence of: 0	D-penicillamine (0.5 mM)
—	29.7 ± 1.9	60.2 ± 1.4
Ca^{2+}, Mg^{2+}	34.0 ± 1.8	62.7 ± 1.6
Ca^{2+}	35.9 ± 1.4	64.3 ± 1.9
Mg^{2+}, EGTA	34.9 ± 1.5	65.1 ± 2.1
BSA	41.7 ± 1.5	74.2 ± 1.7
Ca^{2+}, Mg^{2+}, BSA	47.7 ± 2.0	78.5 ± 1.6
Ca^{2+}, BSA	45.3 ± 1.8	75.4 ± 1.5
Mg^{2+}, EGTA, BSA	46.4 ± 1.6	75.9 ± 1.5

D-Penicillamine was present in the lower compartment of the Boyden chamber only. The other agents were present in both compartments. Ca^{2+}: 1 mM; Mg^{2+}: 1 mM; EGTA: 50 μM; BSA: 0.5% bovine serum albumin.

Values given are the means of three experiments (15 determinations) ± SD.

Table 5. Inhibition of penicillamine-induced locomotion by agents which complex intracellular Ca^{2+}

	Migration (μm) in the presence of: 0	D-penicillamine (0.5 mM)
—	33.3 ± 1.9	63.6 ± 1.1
Quin2-AM (10 μM)	27.3 ± 2.0	34.1 ± 2.2
Chlortetracycline (100 μM)	22.3 ± 1.1	26.2 ± 1.9

Cells were preincubated without or with quin2-AM or chlortetracycline for 10 min at 37°, and subsequently placed in the upper compartment of the Boyden chamber. In the lower compartment 0.5 mM D-penicillamine was placed, and migration was allowed to take place for 40 min. No divalent cations were present during this experiment.

Values given are the means of three experiments (15 determinations) ± SD.

When intracellular Ca^{2+} was complexed by agents which can enter into the cell (quin2, chlortetracycline [18, 19]), spontaneous locomotion was slightly inhibited, but the enhancing effect of penicillamine had disappeared completely (Table 5).

The cellular cGMP concentration strongly increased after application of penicillamine to the cells. The response was rapid, and the cGMP concentration remained elevated as compared with that of control cells, after an initial strong increase (Fig. 3).

Some other compounds with a known activating effect on guanylate cyclase, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and sodium nitroprusside were tested with regard to their ability to increase PMN migration. Applied in the lower compartment of the Boyden chamber 40 μM MNNG increased migration with 89 ± 6%, while 1 mM sodium nitroprusside gave an enhancement of 22 ± 5%.

DISCUSSION

D-Penicillamine has a strong effect on locomotion of PMNs. The experimental procedure determines however, how far that effect is measured. Several authors reported that D-penicillamine had little if any effect on PMN chemotaxis. We could confirm these experiments if the effect of D-penicillamine on

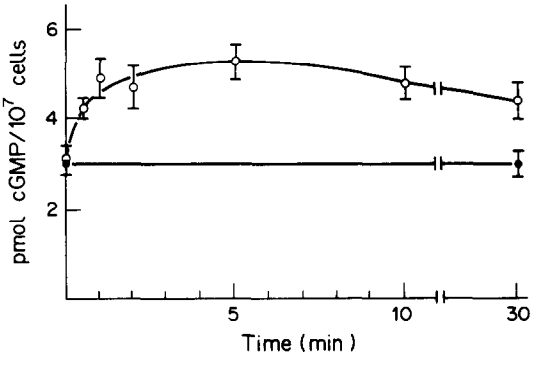

Fig. 3. Effect of penicillamine on cGMP level in rabbit PMNs. Cells (2×10^7 per mL) were exposed to 0.5 mM penicillamine at 37° for the time indicated. Subsequently reactions were stopped by adding perchloric acid, and cGMP level was determined as described in Methods. (●) Control cells; (□) penicillamine-treated cells. Values given are the means of three experiments.

migration towards another strong chemotactic agent was measured, and when D-penicillamine was present in the same compartment of the Boyden chamber as the cells. When, however, sub-optimal concentrations of the other agent (fMet-Leu-Phe) were used, a potentiation was observed.

The data from the literature [3, 6–9], and our own results, indicate that the observation of a potentiation by penicillamine depends on the experimental procedure. A comparable observation was made by investigators who studied the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) on PMN migration [20, 21]. Depending on the location and the pretreatment with GM-CSF either inhibitory or stimulatory effects were observed.

The stimulating effect of D-penicillamine is for a considerable part a chemokinetic effect, but there is also a chemotactic effect. This is indicated by the observation that stimulation was more pronounced if D-penicillamine was in the lower compartment only than when it was present in both compartments of the Boyden chamber. A definite proof of a chemotactic effect in addition to a chemokinetic effect is provided by the checker board assay.

D-Penicillamine-stimulated migration is completely inhibited by sulfhydryl reagents which have little or no membrane permeability. The sulfhydryl reagents affect only the stimulated locomotion, because there is little effect on random locomotion. The results indicate that for the stimulating effect of D-penicillamine on PMN locomotion intact sulfhydryl groups on the outer surface of the plasma membrane are required. The difference between the effect of DTNB pretreatment for penicillamine versus fMet-Leu-Phe-activated migration suggests that the sulfhydryl groups which react with DTNB are of more importance for penicillamine-activated migration than for fMet-Leu-Phe-activated migration. This points to a certain resemblance between the effects of glutathione and penicillamine. We found that both reduced and oxidized glutathione had a strong chemokinetic and a moderate chemotactic effect. Glutathione-enhanced PMN migration could be also completely blocked by DTNB pretreatment. Another resemblance comes from the work of Oben and Foreman [10] who found that both glutathione and D-penicillamine were able to enhance chemotactic peptide-induced exocytosis and the phagocytic uptake of bacteria.

D-Penicillamine forms mixed disulphides with albumin [22]. Albumin is present during the experiments, and it has a chemokinetic effect. The enhancing effect of penicillamine on migration is not due to the formation of mixed disulfides with albumin, because the effect persists in the absence of albumin.

Neither extracellular Ca^{2+} nor Mg^{2+} are absolutely required for PMN locomotion, and their presence has no effect on the enhancement of locomotion by penicillamine. When intracellular Ca^{2+} is removed, the enhancing effect disappears completely. Apparently intracellular Ca^{2+}, but not extracellular Ca^{2+}, is required for the enhancing effect of the drug on PMN locomotion.

Cells exposed to penicillamine show an enhancement of intracellular cGMP level. There are some indications that cGMP is involved in PMN locomotion. Agents such as vitamin c, cholinergic agents and some drugs which enhance cGMP level also enhance PMN locomotion [23–26]. Glutathione too gives an enhancement of cGMP levels. Guanylate cyclase is activated by nitric oxide which is generated by activated PMNs. Chemotaxis is impaired by blocking nitric oxide synthesis and is restored by cGMP derivatives [27]. Some compounds such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and sodium nitroprusside, which are known activators of guanylate cyclase [28, 29], also enhance PMN migration. It seems therefore possible that the effect of penicillamine on PMN locomotion might be related to its effect on cGMP level. The effects of penicillamine in different cell batches was fairly reproducible. Though penicillamine gave always an enhancement of cGMP level, the initial value of cGMP in resting cells, the percentage increase of that level after addition of penicillamine, and its time course varied considerably in different batches. It seems therefore that there is no simple relation between the effect of penicillamine on migration and the enhancement of cGMP level. The exact nature of the relationship thus remains to be determined.

It is not clear whether the effect of penicillamine on PMN migration has any relevance for its effect as an anti-rheumatic agent. Apart from the fact that rabbit peritoneal PMNs differ in some respects from human PMNs, the experimental procedure that is followed to demonstrate the activating effect on migration is quite different from *in vivo* situations. Furthermore, one should expect that agents which allow a faster PMN migration, and thus a faster PMN accumulation, promote inflammation instead of inhibiting it. The ability of penicillamine to activate cGMP level is probably also applicable to other cell systems. Though the role of cGMP is far from clear it is conceivable that the effect on cGMP is related to some of the side-effects of penicillamine.

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