Inhibition of Chemotaxis With N⁶-Monomethyl-L-Arginine: A Role for Cyclic GMP


The metabolism of L-arginine to nitric oxide (NO) has been shown to be important for the effector functions of many cell types, including polymorphonuclear (PMN) leukocytes. Its effect appears to be mediated at least in part by NO stimulation of soluble guanylate cyclase. We evaluated the role of this pathway in two PMN effector functions: cell movement and microbial killing, using the competitive inhibitor L-arginine conversion to NO, N⁶-monomethyl-L-arginine (NMA). We also evaluated the effect of additional L-arginine and dibutyryl cyclic guanosine monophosphate (cGMP) on any NMA-associated changes. Human peripheral blood neutrophils were used and the cells were incubated with and without NMA. Chemotaxis was evaluated using a 48-well micro-Boyden chamber. Microbial killing was evaluated using S aureus strains D2C and 502A. These studies demonstrated that chemotaxis to formyl-methionyl-leucyl-phenylalanine was markedly inhibited in NMA-treated cells. This inhibition could be overcome if L-arginine or dibutyryl cGMP were added with the NMA. In contrast, microbial killing of S aureus was unaffected by NMA. These observations support the hypothesis that the L-arginine metabolism to NO and its effect on the cGMP level may be important for the dynamic changes required for neutrophil chemotaxis.

METABOLISM OF L-ARGININE to nitric oxide (NO) has recently been demonstrated in polymorphonuclear (PMN) leukocytes and a number of other cell types such as macrophages, endothelial cells, and brain. In macrophages, studies in vitro using the potent competitive inhibitor N⁶-monomethyl-L-arginine (NMA) have shown that this pathway is involved in fungistatic activity and tumor cell cytotoxicity. Hibbs et al and Stuehr and Nathan have demonstrated that the macrophage cytotoxic effector molecule is the reactive radical NO, derived from L-arginine. Billiar et al, studying Kupffer cell hepatocyte interactions, have shown that nitrogen oxides inhibit hepatocyte protein synthesis. In endothelial cells, NO was shown to be identical to the endothelium-derived relaxing factor that causes the relaxation of vascular smooth muscle by activating guanylate cyclase. Recently, Brune and Lapetina, studying the NO effect on platelets, reported activation of a cytosolic ADP-ribosyltransferase that occurred independent of guanylate cyclase activation. Although the metabolism of L-arginine in PMNs was described as another source of endothelium-derived relaxing factor with its effect directed at the vascular wall, it was of some interest to determine whether or not products of the metabolism of L-arginine also play a role in other effector functions of PMNs, such as microbial killing and chemotaxis. This was of particular interest since in earlier studies PMNs also had been shown to have a cyclic guanosine monophosphate (cGMP)-mediated response. We now report a marked inhibition of chemotaxis to formyl-methionyl-leucyl-phenylalanine (fMLP) in the presence of NMA. This inhibition could be abrogated both by L-arginine and dibutyryl cGMP, suggesting that the action of NO on PMNs is through cGMP and that endogenous NO production is critical to normal increases in cGMP, which in turn governs chemotaxis.

MATERIALS AND METHODS

Cells. PMN leukocytes were purified from heparinized whole blood by a modification of Boyum's technique with NH₄Cl lysis of red blood cells (RBC) for studies of chemotaxis and orientation as previously described. Leukocytes were resuspended in modified Krebs Ringer phosphate buffer pH 7.4 with 0.8 mg/mL glucose (KRPG) and 0.05% bovine serum albumin. For studies of microbial activity, the PMNs were suspended in KRPG containing 10% autologous serum instead of bovine serum albumin.

Reagents. Unless otherwise stated, these reagents were obtained from Sigma Chemical Co (St Louis, MO). NMA acetate was prepared in our laboratories using a modification of the technique described by Corbin and Reporter. Briefly in these modifications, L-arginine, chelated with CuSO₄, was reacted with N,S-dimethylthiopseudouronium iodide. The copper was precipitated with H₂S and the CuS was removed by filtration. The NMA flavianate was dissolved in hot 20% acetic acid and the flavianic acid removed with Dowex AG-1X2 ion exchange resin. Purity was greater than 99% as assessed with high pressure liquid chromatography. NMA was used at a final concentration of 1 mmol/L. L-arginine was prepared in water and added at concentrations of 1 to 10 mmol/L. Dibutyryl cGMP was prepared in water at a concentration of 1.0 mmol/L and used at a concentration of 0.1 μmol/L.

Chemotaxis. Chemotaxis was measured using a Micro-Boyden chamber modified for the optimal study of neutrophil migration. Briefly, the upper chamber contained 1 × 10⁶ PMNs, and the lower chamber contained 0.1 μmol/L or 0.01 μmol/L fMLP prepared as previously described. Random migration was assessed with high pressure liquid chromatography. NMA was used at a concentration of 0.1 μmol/L.

Bacterial killing. Microbicidal activity was determined as previously described using two strains of S aureus D2C and 502A.

From the Departments of Pathology, Surgery, and Microbiology, Biochemistry, and Molecular Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA.

Submitted July 13, 1989; accepted August 8, 1989.

Partial support provided by the Pathology Education and Research Foundation, and Grant Nos. AI-14032 and GM-37753 awarded by the National Institutes of Health.

Address reprint requests to Sandra S. Kaplan, MD, Department of Pathology, Magee Womens Hospital, Forbes Ave at Halket St, Pittsburgh, PA 15213.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1989 by Grune & Stratton, Inc.

0006-4971/89/7406-0062$3.00/0
INCUBATION OF PMNS WITH 1.0 MMOL/L NMA (OR WITHOUT NMA AS A CONTROL) RESULTED IN A MARKED INHIBITION OF CHEMOTAXIS. CONSIDERING 10 EXPERIMENTS AND USING 0.1 MMOL/L FMLP AS CHEMOATRACTANT, THE CHEMOTAXIS WAS REDUCED TO 52.5 ± 5.2 PERCENT OF THE CONTROL (± SEM). CHEMOTAXIS WAS SIMILARLY REDUCED TO 50.1 ± 6.6 PERCENT OF CONTROL USING 0.01 MMOL/L FMLP. (P < .001 IN BOTH CASES.) RANDOM CELL MOVEMENT WAS NOT SIGNIFICANTLY AFFECTED (74.5 ± 16 PERCENT OF CONTROL). THIS INHIBITION OF CHEMOTAXIS COULD BE PARCELLY OVERCOME BY INCLUDING EITHER L-ARGININE OR CGMP IN THE INCUBATION MEDIUM. WHEN L-ARGININE CONCENTRATIONS OF 1 MMOL/L AND 5 MMOL/L WERE INCLUDED IN THE INCUBATION MEDIUM, CHEMOTAXIS IMPROVED TO 79% TO 91% OF THE PARALLEL CONTROL THAT CONTAINED L-ARGININE BUT LACKED NMA. ALTHOUGH IMPROVED CHEMOTAXIS ALSO WAS SEEN WITH 10 MMOL/L L-ARGININE, THE RESULT DID NOT SHOW STATISTICAL SIGNIFICANCE (TABLE I). THE EFFECT OF 0.1 MMOL/L DIBUTYRYL CGMP ON CHEMOTAXIS IS SHOWN IN TABLE 2. CHEMOTAXIS TO 0.1 MMOL/L FMLP IMPROVED FROM 53.8 ± 3.4 TO 88.8 ± 7.2 PERCENT OF CONTROL (P < .01), AND CHEMOTAXIS TO 0.01 MMOL/L FMLP IMPROVED FROM 66.0 ± 4.1 TO 93.0 ± 6.0 PERCENT OF CONTROL (P < .02).

**Table 1. Effect of L-Arginine on N⁰-Monomethyl-L-Arginine Inhibition of Chemotaxis**

<table>
<thead>
<tr>
<th>FMLP Concentration (µmol/L)</th>
<th>L-Arginine Concentration (mmol/L)</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>58.8 ± 4.1†</td>
<td>88.0 ± 6.3‡</td>
<td>90.5 ± 3.1§</td>
<td>86.8 ± 12.7</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>62.5 ± 3.5†</td>
<td>88.8 ± 3.3‡</td>
<td>79.3 ± 5.0§</td>
<td>89.8 ± 16.2</td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as a mean ± SEM percent of the control chemotaxis determined in the absence of 1 mmol/L NMA, but containing the indicated concentration of L-arginine (n = 4).

*P < .001.
†The control here lacks both L-arginine and NMA. Data reflect the inhibition of chemotaxis associated with NMA.
‡P < .01.
§P < .02.
| | | | | | | |

RESULTS

Incubation of PMNs with 1.0 mmol/L NMA (or without NMA as a control) resulted in a marked inhibition of chemotaxis. Considering 10 experiments and using 0.1 µmol/L FMLP as chemoattractant, the chemotaxis was reduced to 52.5 ± 5.2 percent of the control (± SEM). Chemotaxis was similarly reduced to 50.1 ± 6.6 percent of control using 0.01 µmol/L FMLP. (P < .001 in both cases.) Random cell movement was not significantly affected (74.5 ± 16 percent of control). This inhibition of chemotaxis could be partially overcome by including either L-arginine or cGMP in the incubation medium. When L-arginine concentrations of 1 mmol/L and 5 mmol/L were included in the incubation medium, chemotaxis improved to 79% to 91% of the parallel control that contained L-arginine but lacked NMA. Although improved chemotaxis also was seen with 10 mmol/L L-arginine, the result did not show statistical significance (Table I). The effect of 0.1 µmol/L dibutylryl cGMP on chemotaxis is shown in Table 2. Chemotaxis to 0.1 µmol/L fMLP improved from 53.8 ± 3.4 to 88.8 ± 7.2 percent of control (P < .01), and chemotaxis to 0.01 µmol/L fMLP improved from 66.0 ± 4.1 to 93.0 ± 6.0 percent of control (P < .02).

**Table 2. Effect of Dibutylryl Cyclic GMP on N⁰-Monomethyl-L-Arginine Inhibition of Chemotaxis**

<table>
<thead>
<tr>
<th>FMLP Concentration (µmol/L)</th>
<th>Dibutylryl Cyclic GMP</th>
<th>Absent</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>53.8 ± 3.4*</td>
<td>88.8 ± 7.2‡</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>66.0 ± 4.1*</td>
<td>93.0 ± 6.0‡</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as a mean ± SEM percent of the control chemotaxis determined in the absence of NMA, but containing cGMP (n = 3).

*The control here lacks both cGMP and NMA. Data reflect the NMA associated inhibition of chemotaxis.
†P < .01.
‡P < .02.

Although one product of L-arginine metabolism is the reactive radical NO, which has been shown to participate in macrophage microbiostatic activity, we saw no effect on PMN microbial killing in the presence of NMA. Microbial killing in the presence of NMA was 95% to 100% of the killing when NMA was not present.

DISCUSSION

This study has demonstrated a reversible inhibition of chemotaxis in PMNs exposed to the competitive inhibitor of L-arginine metabolism to NO, NMA. This inhibition of chemotaxis could be prevented if L-arginine or cGMP were present during the incubation with NMA.

The transduction pathway from receptor occupancy to chemotaxis in PMNs is incompletely understood. PMN chemotaxis is achieved by the engagement of specific surface membrane receptors, resulting in a reorganization of the cytoskeleton and movement in the direction of the stimulus. Several groups have reported that chemotaxis was enhanced by substances that stimulated guanylate cyclase and thus raised cGMP. Conversely, PMNs were inhibited by substances that raised cyclic adenosine monophosphate. Stephens and Snyderman also reported this relationship of cGMP to human monocyte chemotaxis. Smith and Ignarro described a similar role for cGMP in stimulus secretion coupling. However, little has recently been said about the role of cGMP in PMN function, and the induction of chemotaxis has been presumed to be derived from the group of transduction reactions that lead to superoxide production.

Interest in cGMP revived when the relaxing factor produced by endothelial cells was shown to be NO derived from the metabolism of L-arginine because this substance was associated with increased production of cGMP. These data supported earlier studies reported by several groups who demonstrated that activation of soluble guanylate cyclase is due to the binding of NO to the heme moiety of guanylate cyclase. We have now demonstrated that inhibition of the metabolism of the L-arginine with NMA was associated with impaired chemotaxis. The abrogation of this impairment by L-arginine suggests that, in fact, the metabolism of L-arginine may be critical for cell movement. The observation that dibutylryl cGMP also prevents NMA-associated inhibition of chemotaxis suggests that the stimulation of soluble guanylate cyclase by NO is important for directional cell movement. Although Brune and Lapetina showed that, in platelets, NO exerts its effect through its
ACTION on soluble ADP-ribosyltransferase without involvement of cGMP, this does not appear to be the explanation in PMNs.

Although L-arginine metabolism was found to be associated with inhibition of fungal growth by macrophages, we failed to see an NO-dependent requirement for bacterial killing by PMNs. This may be because the quantities of NO produced by PMNs are very small compared with macrophages. In addition, neutrophils possess potent microbicidal mechanisms mediated by the oxidative burst and degranulation. We do not propose that this subject is closed, however, since it is highly likely that PMNs have multiple alternative mechanisms of killing in view of the partial killing seen in chronic granulomatous disease leukocytes. The fact that PMNs.

REFERENCES

Inhibition of chemotaxis Ng-monomethyl-L-arginine: a role for cyclic GMP

SS Kaplan, T Billiar, RD Curran, UE Zdziarski, RL Simmons and RE Basford

Updated information and services can be found at:
http://www.bloodjournal.org/content/74/6/1885.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml