K562 Cells Produce an Anti-Inflammatory Factor That Inhibits Neutrophil Functions In Vivo

By M. Amar, N. Amit, J.Y. Scoazec, C. Pasquier, C. Babin-Chevaye, T. Pham Huu, and J. Hakim

We have previously reported that K562, a chronic myelogenous leukemia cell line, releases a low molecular weight factor (6 to 8 Kd) that inhibits human polymorphonuclear neutrophil (PMN) adherence and adherence-related functions tested in vitro. We now report that this factor, which we have named K562 inhibitory factor (K562-IF), has potent anti-inflammatory activity in mice, associated with an inhibition of PMN functions. Its in vitro actions were less marked with mouse PMN than with human PMN. They included (1) an inhibition of both nonstimulated locomotion and locomotion induced by FMLP or serum; (2) an inhibition of the chemiluminescence induced by opsonized zymosan, but not that induced by phorbol myristate acetate or FMLP; (3) an inhibition of the degranulation stimulated by opsonized zymosan, as reflected by lactoferrin and lysozyme release; and (4) a decrease in arachidonic acid release and leukotriene B4 production by A23187-stimulated PMN. The in vivo actions of K562-IF after intraperitoneal injection included (1) an inhibition of subcutaneous PMN accumulation at the site of injection of opsonized zymosan (PMN accumulated neither outside the vessels nor intravascularly, as shown by means of histochemistry); (2) an inhibition of neutrophil accumulation in the peritoneum of mice having received sodium caseinate or opsonized zymosan intraperitoneally; and (3) lysozyme concentration in neutrophils having reached the peritoneum after opsonized zymosan treatment equal to that in blood, suggesting diminished release. PMN influx and degranulation in the peritoneum were reduced by 50% after 3 hours of treatment with 1 μg of K562-IF (equivalent to the effect of 120 μg of prednisolone). Taken together, these results show that K562-IF is a potent anti-inflammatory agent that acts by inhibiting PMN functions.

POLYMORPHONUCLEAR neutrophils (PMN) are an essential component of host defenses against invading microorganisms. However, these cells also play a significant role in host tissue damage during some noninfectious inflammatory reactions. Activated by inflammatory signals, blood PMN adhere to the endothelium, and then migrate and accumulate within inflammatory sites where they release arachidonic acid, leukotrienes, and toxic species such as reactive oxygen derivatives and proteolytic enzymes. All these factors may contribute to the onset and maintenance of inflammatory lesions.

Anti-inflammatory agents act via various mechanisms that include a direct, but most often indirect, inhibitory effect on PMN functions. We have previously shown that K562, a chronic myelogenous leukemia cell line, releases a factor designated K562-IF that inhibits adherence-related functions of human PMN in vitro. To extend these in vitro results to in vivo inflammatory reactions, we investigated the effect of a purified K562-IF preparation on mouse PMN and on PMN influx to subcutaneous sites or into the mouse peritoneum in response to opsonized zymosan or sodium caseinate. Our in vitro findings show that K562-IF inhibits mouse PMN locomotion, chemiluminescence (CL), and release of lysozyme and lactoferrin in response to particulate stimulation, although less markedly than with human PMN. This was also the case for arachidonic acid and leukotriene B4 (LTB4) release by PMN stimulated with the calcium ionophore A23187. In vivo findings show that K562-IF has potent inhibitory activities on PMN accumulation and degranulation in inflammatory sites.

MATERIALS AND METHODS

Reagents

Prednisolone, zymosan A, phorbol myristate acetate (PMA), N-formyl-methionyl-leucyl-phenylalanine (FMLP), cytochalasin B, A23187, Micrococcus lysodeikticus, and lysozyme were from Sigma (St Louis, MO). Antibody against human lactoferrin was from Cappel (Organon Technika, Weidijk, Belgium), anti-interleukin-8 (anti–IL-8) antibody from Janssen (Beerse, Belgium), human IL-8 Quantikine from R & D Systems (Minneapolis, MN), O-dianisidine hydrochloride and hydrogen peroxide from Sigma Chimie (La Verpillère, France), and hexadecyltrimethylammonium bromide and Na caseinate from Merck (Darmstadt, Germany). Glutamine-free RPMI 1604 medium was from Flow Laboratoires (Les Ulis, France) and fetal calf serum (FCS) from Eurobio (Les Ulis, France). [3H] arachidonic acid (specific activity, 8.9 TBq/mmol) and the [3H] radioimmunoassay LTB4 kit were from NEN-Du Pont (Boston, MA). Sodium chromate (2 mCi/mL) was from CEA (Saclay, France). BALB/C mice were from Charles River France (St Aubin-les-Elbouefs, France).

Purification of K562-IF

The routine propagation medium for the established K562 cell line was glutamine-free RPMI 1640 tissue culture medium supplemented with 10% FCS as described by Dokhelar et al. K562-IF was purified from the 48-hour culture supernatant using our previously described technique with two new steps that improved purity. Briefly, the original three-step procedure involved 80% ethanol extraction, gel filtration on sephadex G75, and anion-exchange chromatography on a diethyl aminoethyl (DEAE)-trisacryl M column. The two supplementary purification steps were 80% ethanol extraction between the sephadex G75 filtration and DEAE-anion-exchange chromatography steps, and a final cation-exchange chromatography on a carboxy-methyl (CM)-trisacryl M column (0.05 mol/L sodium acetate buffer, pH 5, NaCl gradient 0 to 1 mol/L) of the active fraction eluted from the anion-exchange chromatography step (Table 1). The potency of the active fraction was determined throughout the purification procedure by its...
CANCER-DERIVED INHIBITOR OF PMN IN VIVO

Table 1. Purification of K562-IF

<table>
<thead>
<tr>
<th>Step*</th>
<th>%Shape Change</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>80% Ethanol extraction</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>Sephadex G75 filtration</td>
<td>81</td>
<td>58</td>
</tr>
<tr>
<td>80% Ethanol extraction</td>
<td>65</td>
<td>29</td>
</tr>
<tr>
<td>DEAE-anion exchange</td>
<td>46</td>
<td>16</td>
</tr>
<tr>
<td>CM-cation exchange</td>
<td>40</td>
<td>6</td>
</tr>
</tbody>
</table>

*Supernatant of a 48-hour culture of K562 was used as the starting material for the purification of K562-IF. After extraction with 80% ethanol and solvent evaporation, the resulting residue was suspended in distilled water (first 80% ethanol extract). Purification steps are in Materials and Methods.

†Values shown are FMLP-induced PMN shape changes observed with the various K562 products (at a protein concentration of 0.02 mg/mL) expressed as a percentage of shape changes observed in the absence of K562-IF products.

‡Based on the first ethanol extract as starting material that contains 5 mg of total proteins.

Inhibitory effect on human PMN shape changes, measured according to Smith et al. A

In vitro experiments. Venous blood of mice (7- to 8-week-old Balb/c) and healthy blood donors was collected on preservative-free Li-heparinate (10 IU/mL). Blood from 12 mice was pooled, and PMN were isolated by density gradient centrifugation on Ficoll-Hypaque followed by Dextran sedimentation, as previously described. B After hypotonic lysis of contaminating red blood cells, PMN were washed twice with Ca2+- and Mg2+-free Krebs Ringer phosphate buffer (KRP) at pH 7.4, and resuspended in the same buffer at appropriate concentrations.

Human PMN shape changes were measured according to Smith et al. C Briefly, after incubation for 10 minutes at 37°C with K562-IF or control medium, PMN (10⁶ in 160 μL) were stimulated with FMLP (10⁻⁷ mol/L) for an additional 10 minutes. The reaction was stopped by adding ice-cold 1% glutaraldehyde (vol/vol); the percentage of cells with monopolar or bipolar configurations was determined. Two hundred cells were examined by means of phase-contrast microscopy for each assay (performed in duplicate).

Random and oriented locomotion of PMN were measured as previously described by means of the under-agarose technique. D The PMN were preincubated with K562-IF or KRP. For human and mouse PMN, the attractants used were FMLP (10⁻⁷ mol/L and 10⁻⁸ mol/L, respectively) and human serum. Migration distances were measured, using a calibrated eyepiece, from the leading front of migrating cells (at least 10 PMN) at a magnification of ×40, as the distance traversed by the cells towards the chemotactant wells (oriented migration) or towards the control wells (random migration).

Adherence of PMN was measured on culture plates (Falcon 3072; Microtest Becton Dickinson, Grenoble, France) with 3⁵-Cr-labeled PMN, as previously described. E PMN resuspended in KRP (4 × 10⁶ PMN/mL) were incubated with K562-IF or KRP as described above. PMN suspensions were poured into wells (4 × 10⁶ PMN/well) and incubated for 30 minutes at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Nonadherent cells were removed by washing with 1 mL of buffer and adherent cells were lysed with 0.2% Triton X-100 in 1 mL of buffer. The percentage adherence was calculated as (cpm of adherent cells [cpm of nonadherent cells] + cpm of adherent cells) × 100.

Luminol-enhanced CL was measured as previously described. F The reaction cuvettes contained 0.05 mL of 10⁻⁶ mol/L luminol solution, 0.1 mL of a suspension of mouse or human PMN (10⁶ cells/mL preincubated with K562-IF or KRP for 10 minutes at 37°C) and 0.05 mL of PMA (1 μg/mL), or FMLP (10⁻⁶ mol/L). The cuvettes were placed in a luminometer (Packard Picolite 6500; United Technologies, Packard, Downers Grove, IL) and maintained at 37°C with magnetic stirring. The peak CL response was expressed in cpm/10⁶ PMN.

Degranulation studies were performed on mouse (4 × 10⁶/mL) and human PMN (5 × 10⁶/mL) preincubated for 10 minutes at 37°C with K562-IF or control medium in the presence of cytochalasin B (5 μg/mL). PMN were then stimulated for 20 minutes at 37°C with 0.5 mg/mL of zymosan opsonized with normal human AB serum. The reaction was stopped by placing the tubes in an ice-water bath. The supernatant and cell pellet were recovered after centrifugation (400g) at 4°C for 10 minutes. Lysozyme and lactoferrin were determined by means of a Micrococcus lysodeikticus turbidimetric method G and an enzyme-linked immunosorbent assay (ELISA) with anti-human lactoferrin antibody, respectively. H Degranulation was expressed as micrograms of egg-white lysozyme equivalent and micrograms of lactoferrin per 10⁶ PMN.

Arachidonic acid release was measured with mouse PMN (1 × 10⁶/mL) or human PMN (5 × 10⁶/mL) that had been loaded with 1 μCi of [³⁵]arachidonic acid (8.9 TBq/mmol or 240 Ci/mmol) at 37°C for 90 minutes. PMN were washed three times in cold KRP and resuspended in 1 mL KRP. After preincubation with or without K562-IF for 10 minutes at 37°C, the PMN were then stimulated with 0.5 μmol/L of A23187 at 37°C for 10 minutes. The reaction was stopped by placing the tubes in an ice-water bath. After centrifugation, the supernatants and pellets were collected and radioactivity counted. Arachidonic acid release was calculated as (supernatant cpm [supernatant cpm + pellet cpm]) × 100.

LTB₄ production was measured with mouse PMN (3.2 × 10⁶/mL) or human PMN (5 × 10⁶/mL) preincubated for 10 minutes with K562-IF or control medium and then stimulated for 5 minutes at 37°C with 1 × 10⁻⁶ mol/L A23187. The reaction was stopped by placing the tubes in an ice-water bath. After centrifugation, the supernatant was stored at −70°C until LTB₄ assay using a [³⁵]H LTB₄ RIA kit (NEN-DuPont). Results are expressed in picograms per 10⁶ PMN and per minute.

In vivo experiments. Seven- to 8-week-old BALB/C mice weighing 18 to 20 g were used. Mice received an intraperitoneal (IP) injection of either 0.1 mL of sterile physiologic saline solution (control), 12 μg K562-IF, or 120 μg prednisolone. Fifteen minutes later, subcutaneous inflammation was induced by injection of 0.1 mL of opsonized zymosan (0.3 mg) on both sides of their spinal column. The mice were killed 2 hours after injection. The peripheral blood was collected to measure the myeloperoxidase (MPO) activity of PMN by the O-dianisidine method, as previously described, I and the skin was dissected in the areas of the opsonized zymosan injection. One side was evaluated histologically and the other weighed and assayed for MPO activity.

For histologic examination, subcutaneous samples were processed using conventional techniques. Fragments were fixed in formaldehyde, dehydrated, and included in paraffin. Serial 4-μm sections were cut, stained with hematoxylin-eosin, and examined under a light microscope.

Evaluation of subcutaneous MPO content was performed according to Smith et al. J Tissue samples (150 to 400 mg) were homogenized in 20 mmol/L potassium phosphate buffer (pH 7.4) containing 0.1 mmol/L EDTA using a Potter-Elvehjem tissue homogenizer (Tri-R Instruments Inc, New York, NY) (three times for 30 seconds each, on ice). One milliliter of tissue homogenate was centrifuged at 20,000g for 15 minutes at 4°C. The pellet was then rehomogenized in an equivalent volume of 0.05 mol/L potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. The homogenates were freeze-thawed from www.bloodjournal.org by guest on October 3, 2017. For personal use only.
were tested at a concentration of 0.01 mg/mL, and inhib-
control, sodium caseinate (12 mg/mL), or opsonized zymosan (10 mg/mL). Group I (5 mice) received 0.2 mL of saline excipient; group II (10 mice) received caseinate; group III (11 mice) received caseinate and 120 μg prednisolone; group IV (12 mice) received K562-IF (1 μg) and caseinate; group V (8 mice) received zymosan; group VI (8 mice) received zymosan and 1 μg K562-IF; and group VII (8 mice) received zymosan and 4 μg K562-IF. The exudate was collected 3 hours (in some experiments 6 and 18 hours) after the injection by peritoneal washing with 2 mL of saline. The total number of PMN collected from the peritoneal cavity was calculated by staining a standard cuve that was linear from 3 x 10^3 to 12 x 10^3 PMN.

Peritoneal exudation was induced by IP injection of 0.2 mL of sterile physiologic saline solution (control), sodium caseinate (12 mg/mL), or opsonized zymosan (10 mg/mL). Group I (5 mice) received 0.2 mL of saline excipient; group II (10 mice) received caseinate; group III (11 mice) received caseinate and 120 μg prednisolone; group IV (12 mice) received K562-IF (1 μg) and caseinate; group V (8 mice) received zymosan; group VI (8 mice) received zymosan and 1 μg K562-IF; and group VII (8 mice) received zymosan and 4 μg K562-IF. The exudate was collected 3 hours (in some experiments 6 and 18 hours) after the injection by peritoneal washing with 2 mL of saline. The total number of PMN collected from the peritoneal cavity was calculated by staining a standard cuve that was linear from 3 x 10^3 to 12 x 10^3 PMN.

Statistical Analysis

Results are given as mean ± SD for a given number of experiments. Differences were analyzed using the Student’s t-test and the level of significance was set at P < .05.

RESULTS

Characterization of K562-IF

The previous preparation of K562-IF used at 0.2 mg protein/mL produced 45% to 55% inhibition of human PMN locomotion and shape changes induced by FMLP in vitro. By comparison, at a concentration of 0.02 mg/mL (Table 1), the purified K562-IF preparation inhibited 60% of FMLP-induced shape changes, indicating that its inhibitory activity was 10-fold higher than that previously obtained by the initial three-step procedure. The more active K562-IF preparation was used in this study. Figure 1 shows the results of analysis by high-performance liquid chromatography (HPLC) on an UltraPac TSK 125 column (Bio Rad, Ivry sur Seine, France) in 0.05 mol/L phosphate buffer, pH 6.8. The two fractions (F1 and F2) of the peak were tested at a concentration of 0.01 mg/mL, and inhibited 43% and 93% of FMLP-induced shape changes, respectively. A better separation of the two fractions should allow us to define their biochemical composition and to understand why each fraction exhibits a much larger activity than that of their mixture.

Effect of K562-IF on Mouse PMN Functions In Vitro

Before testing the possible anti-inflammatory effect of K562-IF in mice, we evaluated its in vitro efficiency on mouse PMN in comparison to that on human PMN.

PMN locomotion. The purified K562-IF preparation at a concentration of 0.02 mg/mL inhibited both nonstimulated locomotion of human PMN (by about 75%) and locomotion induced by either FMLP or serum (73% and 84% inhibition, respectively) (Table 2). K562-IF was less effective on mouse PMN locomotion (50% inhibition). K562-IF used as chemoattractant instead of serum, or FMLP was effective on neither human PMN, as previously reported, nor on mouse PMN. Mixed with either FMLP or serum, K562-IF did not alter chemotactic activity, suggesting that it inhibited mouse PMN locomotion by acting on the cell and not on the chemoattractant. The inhibitory effect of K562-IF on mouse PMN locomotion persisted when PMN were washed after being incubated with the factor, as previously reported for human PMN.

PMN adherence. The purified K562-IF preparation inhibited both human and mouse PMN adherence (75% and 20% inhibition, respectively). K562-IF was again less effective on mouse PMN (Table 2).

Luminol-enhanced CL of stimulated PMN. Table 2 shows the effect of K562-IF on mouse and human PMN CL response induced by FMLP, PMA, and opsonized zymosan. The CL response of mouse and human PMN pretreated by K562-IF was decreased when PMN were stimulated by opsonized zymosan, whereas it was not altered when they were stimulated by PMA or FMLP. These results show that this more purified K562-IF preparation was effective on the oxidative burst induced by a particulate stimulant, whereas it did not alter that induced by soluble stimuli.

Lysozyme and lactoferrin release by PMN. K562-IF inhibited the release of lysozyme and lactoferrin by human and mouse PMN stimulated with opsonized zymosan (Table 2). K562-IF was less effective on mouse PMN than on human PMN.

Arachidonic acid release and LTB4 generation by PMN. As shown in Table 3, K562-IF inhibited mouse and human PMN arachidonic acid release after stimulation with the calcium ionophore A23187. A similar inhibitory effect of K562-IF on LTB4 synthesis by PMN stimulated with A23187 was observed (Table 3).

Effect of K562-IF on the Inflammatory Response Induced in the Mouse by Subcutaneous Injection of Opsonized Zymosan

MPO activity was measured in tissue samples at the site of subcutaneous injection of opsonized zymosan in animals.
that received an IP injection of saline solution, prednisolone, or K562-IF (Table 4). Both K562-IF and prednisolone decreased MPO, by 77% and 79%, respectively.

Histologic examination of control animals showed zymosan deposits located at variable depths within the hypodermis and surrounded by large numbers of PMN, which formed a thick ring around the injected material (Fig 2a). Most PMN contained phagocytosed zymosan particles (Fig 2c). Infiltration of the hypodermis by PMN was prominent and visible even at some distance from the injected material (Fig 2a and c). Surrounding vessels also contained large numbers of PMN. Images suggestive of leukocyte adhesion and diapedesis were readily observed (Fig 2c). In K562-IF-treated animals, as in prednisolone-treated animals (data not shown), zymosan deposits were surrounded by a thin and often incomplete ring of PMN (Fig 2b), and most of the PMN in contact with zymosan contained phagocytosed material (Fig 2d). The number of extravasated PMN in the surrounding hypodermis was lower than in control animals (Fig 2d). Surrounding vessels appeared empty or contained only small numbers of PMN (Fig 2d). Images suggestive of leukocyte adhesion and diapedesis were rare. Histologic examination confirmed that the numbers of PMN present in the inflammatory site were far lower in K562-IF–treated mice than in untreated controls, but did not indicate whether the anti-inflammatory effect concerned PMN adhesion to vessel walls only or concerned both adhesion and diapedesis.

Effect of K562-IF on the Inflammatory Response Induced in the Mouse Peritoneum by Opsonized Zymosan or Sodium Caseinate

As expected, 3 hours after an IP injection, opsonized zymosan or sodium caseinate induced a much larger accumulation of PMN in the peritoneum than that obtained with saline (Table 5). The amount of liquid recovered after

<table>
<thead>
<tr>
<th>Table 2. Effect of K562-IF on Human and Mouse PMN</th>
<th>Control PMN (U)</th>
<th>K562-IF–Treated PMN (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Human</td>
<td>Mouse</td>
</tr>
<tr>
<td>Locomotion* (4)</td>
<td>0.25 ± 0.08</td>
<td>0.35 ± 0.2</td>
</tr>
<tr>
<td>FMLP</td>
<td>0.56 ± 0.15</td>
<td>1.78 ± 0.25</td>
</tr>
<tr>
<td>Human LTB4 (3)</td>
<td>0.90 ± 0.14</td>
<td>1.33 ± 0.28</td>
</tr>
<tr>
<td>Adherence* (4)</td>
<td>49.4 and 44.7</td>
<td>44.0 ± 12 (4)</td>
</tr>
<tr>
<td>CLS(4)</td>
<td>0.14 ± 0.27</td>
<td>2.20 ± 0.15</td>
</tr>
<tr>
<td>PMA</td>
<td>4.15 ± 0.02</td>
<td>1.36 ± 0.41</td>
</tr>
<tr>
<td>FMLP</td>
<td>0.65 ± 0.12</td>
<td>1.75 ± 0.18</td>
</tr>
<tr>
<td>OZ</td>
<td>0.75 ± 0.02</td>
<td>3.00 ± 0.35</td>
</tr>
<tr>
<td>ZO-induced release (4)</td>
<td>2.0 ± 0.00</td>
<td>5.4 ± 1.2</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>1.6 ± 0.7</td>
<td>3.8 ± 0.7</td>
</tr>
</tbody>
</table>

Number of experiments are given in parentheses. Results for K562-IF–treated PMN are expressed as a percentage of the paired controls.

*Random locomotion and FMLP: serum-induced locomotion were evaluated by an under-agarose migration technique. Results for control migrations are given in millimeters.

†Significant differences (P < .05) between controls and K562-IF–treated PMN.

PMN adherence was measured as described in Materials and Methods. Results are given in percent of cells that adhered for controls and in percent of controls for K562-IF–treated PMN. The two results obtained in mice are given, whereas mean ± 1 SD is given for humans.

§The peak of luminol-enhanced CL response of control PMN to PMA (1 μg/mL) or IP injection of saline solution, prednisolone (2 mg/mL), or opsonized zymosan (OZ) (2 mg/mL) are expressed in 10⁴ cpm/10⁵ PMN.

<p>| Table 3. Effect of K562-IF on Release of [3H] Arachidonic Acid and LTB4 Generation by PMN |</p>
<table>
<thead>
<tr>
<th>Control PMN (U)</th>
<th>K562-IF–Treated PMN (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Human</td>
</tr>
<tr>
<td>Arachidonic acid*</td>
<td>6.59 ± 0.7</td>
</tr>
<tr>
<td>Buffer (3)</td>
<td>12.5 ± 0.2</td>
</tr>
<tr>
<td>LTBI†</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>A23187 (3)</td>
<td>7.5 ± 2.9</td>
</tr>
</tbody>
</table>

The number of experiments, each performed in duplicate, is given in parentheses.

Abbreviation: ND, not done.

*Mouse PMN (1 × 10⁶) or human PMN (5 × 10⁶) loaded with [3H] arachidonic acid were resuspended with or without (controls) 20 μg/mL K562-IF. PMN were then stimulated with 0.5 μmol/L or A23187. Supernatants and cell pellets were separated and radioactivity was counted in each fraction. Results of arachidonic release in control PMN are expressed in percentage of total [3H] arachidonic acid. In K562-IF–treated PMN results are in percentage of release measured in paired controls.

†Significant differences (P < .05) between controls and K562-IF–treated PMN.

Mouse PMN (3 × 10⁶) or human PMN (5 × 10⁶) preincubated with buffer or K562-IF were stimulated with 1 μmol/L A23187 for 5 minutes at 37°C. LTB4 was determined in the supernatants (see Materials and Methods for technical details). Results are expressed in picograms in per 10⁵ PMN per minute for the controls and in percentage of paired controls for K562-IF–treated PMN.

<table>
<thead>
<tr>
<th>Table 4. Effect of K562-IF on the Subcutaneous Inflammation Induced by Opsonized Zymosan</th>
<th>Control PMN (U)</th>
<th>K562-IF–Treated PMN (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Human</td>
<td>Mouse</td>
</tr>
<tr>
<td>Arachidonic acid*</td>
<td>6.59 ± 0.7</td>
<td>8.85 ± 0.65</td>
</tr>
<tr>
<td>Buffer (3)</td>
<td>12.5 ± 0.2</td>
<td>15.2 ± 0.15</td>
</tr>
<tr>
<td>LTBI†</td>
<td>0.2 ± 0.1</td>
<td>4.0 ± 3.4</td>
</tr>
<tr>
<td>A23187 (3)</td>
<td>7.5 ± 2.9</td>
<td>52.4 ± 14.8</td>
</tr>
</tbody>
</table>

The number of experiments, each performed in duplicate, is given in parentheses.

Abbreviation: ND, not done.

*Mouse PMN (1 × 10⁶) or human PMN (5 × 10⁶) loaded with [3H] arachidonic acid were resuspended with or without (controls) 20 μg/mL K562-IF. PMN were then stimulated with 0.5 μmol/L or A23187. Supernatants and cell pellets were separated and radioactivity was counted in each fraction. Results of arachidonic release in control PMN are expressed in percentage of total [3H] arachidonic acid. In K562-IF–treated PMN results are in percentage of release measured in paired controls.

†Significant differences (P < .05) between controls and K562-IF–treated PMN.

Mouse PMN (3 × 10⁶) or human PMN (5 × 10⁶) preincubated with buffer or K562-IF were stimulated with 1 μmol/L A23187 for 5 minutes at 37°C. LTB4 was determined in the supernatants (see Materials and Methods for technical details). Results are expressed in picograms in per 10⁵ PMN per minute for the controls and in percentage of paired controls for K562-IF–treated PMN.
washing the peritoneum with normal saline was about 10% less than that injected (2 mL) and the ascitic response to sodium caseinate and zymosan could not therefore be quantified. The coadministration of K562-IF (1 μg per mouse) significantly reduced PMN accumulation in the peritoneum after injection of the two stimuli. The effect was similar to that obtained with 120 μg of prednisolone (Table 5). It was also dose-related, because 4 μg of K562-IF was more effective than 1 μg, completely abolishing PMN influx. K562-IF was also inhibitory for PMN accumulation in the peritoneal cavity of mice, 6 and 18 hours after initiation of the inflammatory response (Table 5). However, the inhibitory effect decreased with time, suggesting an in vivo liability of K562-IF. It is noteworthy that IP injection of K562-IF increased blood neutrophil counts by about 40% (3 hours after its injection) in zymosan-treated or untreated mice.

To assess whether the PMN that reached the peritoneum in K562-IF-treated mice could mount a second inflammatory response, ie, degranulation, we determined cellular lysozyme content. Lysozyme in resting PMN is located in specific and azurophilic granules and can be released into the extracellular medium in response to stimulation. We found that peritoneal PMN contained much more lysozyme when the mice had been treated with zymosan and K562-IF than with zymosan alone, as would be expected from decreased degranulation (Table 6). Furthermore, the lysozyme content of PMN recovered 3 hours after saline

### Table 5. Effect of K562-IF on the Number of PMN Recovered in the Peritoneal Exudate Induced in Mice by IP Injection of Na Caseinate or Opsonized Zymosan

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Total No. of PMN (mean ± SD [× 10^6]) After 3 h</th>
<th>6 h</th>
<th>18 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Saline (5)</td>
<td>232 ± 60</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>II</td>
<td>Na caseinate (10)</td>
<td>694 ± 66</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>III</td>
<td>Na caseinate + prednisolone (11)</td>
<td>340 ± 39*</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>IV</td>
<td>Na caseinate + 4 μg K562-IF (12)</td>
<td>230 ± 70*</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>V</td>
<td>Zymosan (8)</td>
<td>455 ± 80</td>
<td>596 ± 64</td>
<td>930 ± 77</td>
</tr>
<tr>
<td>VI</td>
<td>Zymosan + 1 μg K562-IF (8)</td>
<td>325 ± 88†</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>VII</td>
<td>Zymosan + 4 μg K562-IF (8)</td>
<td>242 ± 50†</td>
<td>290 ± 60</td>
<td>470 ± 26</td>
</tr>
</tbody>
</table>

Group I of mice received saline, group II received Na caseinate, and groups III and IV received Na caseinate plus 120 μg prednisolone or 4 μg K562-IF, respectively. Groups V, VI, and VII received opsonized zymosan and groups VI and VII received 1 and 4 μg K562-IF, respectively. Three and, in some experiments, 6 and 18 hours after treatment, the peritoneal cavity was washed with 2 mL of isotonic saline. Data are mean ± 1 SD of the total number of PMN recovered. The number of mice treated in each group is given in parentheses.

*P < .05 v group II.
†P < .05 v group V.

### Table 6. Effect of K562-IF on Lysozyme Content of PMN Recovered From the Peritoneal Exudate Induced in Mice by IP Injection of Opsonized Zymosan

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Lysozyme Content of 10^6 PMN (in μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>Zymosan (8)</td>
<td>0.82 ± 0.36</td>
</tr>
<tr>
<td>VI</td>
<td>Zymosan + 1 μg K562-IF (8)</td>
<td>0.92 ± 0.32</td>
</tr>
<tr>
<td>VII</td>
<td>Zymosan + 4 μg K562-IF (8)</td>
<td>3.03 ± 1.13*</td>
</tr>
</tbody>
</table>

Conditions and symbols are the same as for Table 5 except that the peritoneal exudate studied were from groups V, VI, and VII only. PMN were harvested, 3, 6, and 18 hours after the injection and their lysozyme content (micrograms per 10^6 PMN) measured. Lysozyme content of peritoneal PMN from group I (saline-treated mice; see Table 5) was 5.34 ± 0.85 3 hours after treatment.
treatment (controls) was 5.34 ± 0.85 μg/10⁶ PMN, ie, similar to that of mice treated with 4 mg of K562-IF (Table 6).

DISCUSSION

The results of this study show that IP administration to mice of K562-IF, a product of malignant cells, has potent antiinflammatory effects in vivo. Indeed, it inhibited subcutaneous PMN accumulation in response to the injection of opsonized zymosan, and both PMN accumulation and degranulation in the peritoneal cavity of mice injected IP with sodium caseinate or opsonized zymosan. Inhibition of subcutaneous PMN accumulation by IP injection of K562-IF was established by measuring the level of tissular MPO activity at the site of tissue injury and by histologic examination. Tissular MPO activity is a specific marker of both intact PMN and those having released their MPO content. In both cases, MPO activity can be considered representative of PMN infiltration. In addition, Krawisz et al10 have reported that the level of MPO activity found in inflamed tissues is directly proportional to the number of neutrophils. Our results clearly show that MPO activity in the subcutaneous tissues of K562-IF–treated mice was less than one-quarter of that in untreated mice. Direct histologic examination of tissue sections confirmed this result and suggested that the decreased PMN accumulation was associated with a decrease in the number of PMN adhering to postcapillary veinules. This is also suggested by the fact that K562-IF increased blood neutrophil counts.

To be able to count the number of PMN reaching the inflamed tissues in K562-IF–treated mice, the inflammatory stimuli were injected IP. The results confirmed that the number of PMN leaving the blood vessels in response to inflammatory stimuli was decreased by K562-IF and that K562-IF acts more efficiently at the site of injection than distally. Moreover, PMN that were found in the peritoneal cavity in K562-IF–treated mice contained sixfold more lysozyme than PMN in untreated animals 3 hours after zymosan injection. The high lysozyme content of PMN recovered in the peritoneum after treatment with opsonized zymosan and K562-IF suggests that they had not degranulated, because it was similar to that of peritoneal PMN recovered from saline controls in terms of both the mean and SD (Table 6). However, we cannot exclude the possibility that K562-IF inhibits extravasation only of PMN with a low lysozyme content. Nonetheless, K562-IF inhibits PMN degranulation in vitro, as shown in terms of both lactoferrin (a marker of secondary granules) and lysozyme (a marker of both primary and secondary granules) release.15

The mechanism by which PMN accumulation in the inflamed sites is inhibited could not be determined. However, it is clear that K562-IF inhibits the adherence, shape changes, and locomotion of mouse PMN in response to FMLP or serum in vitro, and it is thus reasonable to suppose that, in vivo, K562-IF inhibits migration into inflamed tissues. K562-IF also inhibits PMN adherence and could have acted in vivo by hindering their adherence to capillaries adjacent to the site of inflammation. Histologic examination of tissue sections, as well as increased number of circulating PMN, indeed supported such a mechanism. On the other hand, it has been recently reported that the mechanism of the inflammatory activity of zymosan injected IP involves the generation not only of C5a and IL-8–like molecules,16 but also involves LTB₄ production.17 Recruitment of PMN by LTB₄ generated by the first PMN to arrive at the inflammatory site has been reported,18 and our results clearly show that K562-IF inhibits LTB₄ production by stimulated PMN (Table 3). It is thus possible that K562-IF acts indirectly by inhibiting the generation of such chemoattractants. LTB₄ acts not only as a chemoattractant but also as a stimulant of PMN adhesion and migration across the endothelial wall.19,20 The mechanism by which K562-IF inhibits LTB₄ production is not settled. However, it is well known that PMN challenged with A23187 or opsonized zymosan release arachidonic acid from membrane phospholipids mainly through the activation of phospholipase A₂.21 Some of the arachidonic acid produced is converted via the PMN lipooxygenase pathway into leukotrienes such as LTB₄.22,23 K562-IF inhibited not only LTB₄ formation, but also arachidonic acid release, suggesting that the latter effect might be responsible for the former. Further studies are required to determine whether the inhibition of PMN accumulation in inflammatory sites by K562-IF is due to a direct inhibition of PMN adherence and locomotion, an inhibition of the generation of chemoattractants such as LTB₄, or both.

The in vitro inhibitory effect of K562-IF on human PMN reported here is much stronger than that on mouse PMN. If this in vitro difference also exists in vivo, K562-IF could have much more potent anti-inflammatory effects in humans than in mice. This could mean that the release of K562-IF–like molecules by cancer cells may be responsible for the defective inflammatory cell accumulation associated with neoplasias.24 Indeed, defective PMN chemotaxis and adherence has been reported in patients with chronic myeloid leukemia.25 Defective macrophage accumulation in response to various stimuli,26 as well as impaired monocyte function, have also been widely reported in association with neoplasias and tumor growth.27-28 We have shown that K562-IF differs from the previously identified soluble factor that inhibits monocyte/macrophage functions.3 It also differs from the 77 amino acids IL-8 variant whose inhibitory activity on PMN adherence has been reported by some investigators29,30 and questioned by others.31 In fact, IL-8 was undetectable (<2 pg/mL) in K562-IF preparation containing 0.1 mg protein/mL (Quantikine human IL-8 immunoassay), and an anti–IL-8 antibody mixed with K562-IF did not alter its effects on PMN shape changes (results not shown).

In conclusion, our data show that K562-IF, a tumor-derived factor, inhibits mouse PMN functions both in vitro and in vivo. K562-IF also inhibits LTB₄ generation by PMN and might thereby block further PMN recruitment by the first cells to arrive at the inflammatory site. Moreover, we found that those PMN that did reach the inflammatory site...
did not degranulate. K562-IF is thus a potent anti-inflammatory agent that acts on PMN. This could partly explain the ability of neoplasms to depress host resistance to infection and tumor development. Purification of K562-IF is underway with a view to its structural analysis and chemical synthesis. This would open the way to studies on the possible therapeutic use of K562-IF analogues and K562-IF antagonists in humans.

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K562 cells produce an anti-inflammatory factor that inhibits neutrophil functions in vivo

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