A Peroxidase Inhibitor in Leukemic AKR Mouse Spleen Cells

Robert R. Strauss, Benoy B. Paul, Ratnam J. Selvaraj, and Anthony J. Sbarra

Department of Pathology and Medical Research, St. Margaret's Hospital, and Department of Obstetrics and Gynecology, Tufts University School of Medicine, Boston, Massachusetts 02125

SUMMARY

Spleen cell suspensions from leukemic AKR mice, unlike those from normal animals of the same strain, were found to be devoid of in vitro bactericidal activity. These cells were also found to be deficient in glucose 6-phosphate dehydrogenase, hexose monophosphate shunt, and peroxidase activities. In contrast, formate-14C oxidation, a measurement of H2O2, by spleen cells from leukemic animals was markedly elevated when compared to this activity of cells from nonleukemic AKR mice. The 20,000 X g pellet fraction of the spleen cell homogenates from leukemic mice was devoid of peroxidase activity. This fraction was not bactericidal in the presence of H2O2 and chloride ion at acidic pH. Indeed, this high speed pellet of homogenates of leukemic mice markedly inhibited peroxidase-mediated amino acid decarboxylation and bacterial functions of the same fraction from homogenates of spleen cells from nonleukemic control animals. The inhibitory activity was found to be heat stable and nondialyzable.

INTRODUCTION

We have previously reported (22) that spleen cell suspensions from AKR strain mice, predominantly lymphocytes by morphology, are able to kill Escherichia coli in vitro. These cells also exhibit stimulation of a number of metabolic activities, when presented with bacteria or other particles, that are associated with antimicrobial activity in polymorphonuclear leukocytes (18). However, actual engulfment of particulate matter was not demonstrable in spleen cells. Stimulation of SPO activity was notable among the number of metabolic functions found to be increased in the presence of particles. SPO activity was found to be localized primarily in the 20,000 X g pellet fraction of spleen cell homogenates. The presence of chloride ion, sublethal concentrations of H2O2, and pH 5.5, this fraction effectively killed a variety of both Gram-positive and Gram-negative bacteria (23). The peroxidase activity from spleen cell suspensions appears to be qualitatively similar to that of myeloperoxidase. It differs quantitatively in that the polymorphonuclear leukocyte enzyme is more potent on a per guaiacol unit basis (21, 22).

The data to be presented indicate no demonstrable SPO or bactericidal activity in spleen cells obtained from leukemic AKR mice. The purpose of this report is to show that the apparent defect in both bactericidal and peroxidase activities is due to the presence of a peroxidase inhibitor in the particulate fraction of spleen cells from leukemic mice. The possible relationship between those observations and the increased susceptibility to infections experienced by patients with lymphocytic leukemia will be discussed.

MATERIALS AND METHODS

AKR mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, and maintained in the animal facility at St. Margaret's Hospital. Mice were sacrificed by cervical dislocation and spleens were removed aseptically. Spleen cell suspensions were prepared relatively free of red blood cells by previously described procedures (22). Where homogenates were desired homogenation in 0.25 M sucrose was carried out for 2 min at 3800 rpm in a Potter Elvehjem homogenizer with a Teflon-tipped, motor-driven pestle. Centrifugal fractionation of homogenates was carried out in a refrigerated (4°) Servall RC-2 centrifuge.

Bactericidal activity was determined with E. coli from the St. Margaret's Hospital stock culture collection. The organisms were quantitated from a turbidimetric standard curve prepared from a turbidity versus colony count relationship. The bactericidal activity of intact spleen cells was ascertained by incubating equal numbers of bacteria and spleen cells for 60 min at 37° in Kreb-Ringer phosphate buffer, pH 7.4. At the end of the incubation period aliquots were removed from both the bacterial control and the tube containing both cells and bacteria. These aliquots were diluted 1:5 in 0.5% saponin in order to lyse the spleen cells. Subsequent serial logarithmic dilutions were made in 0.9% NaCl solution. Duplicate 0.1-ml portions were taken from appropriate dilutions for plate counts in trypticase soy agar using a semimicro pour plate procedure.

The experiments involving the bactericidal activity of the 20,000 X g pellet fraction of spleen cell homogenates utilized the same strain of E. coli in the logarithmic growth phase. Each tube contained 1 X 10^8 bacteria per ml. A typical experiment contained 4 tubes: a bacterial control; 5 X 10^{-5} M H2O2 + bacteria; 20,000 X g pellet fraction containing 0.03 guaiacol unit of peroxidase activity + bacteria; and the complete system consisting of H2O2, peroxidase containing pellet, and bacteria. The reactions were conducted in...
Krebs-Ringer phosphate buffer, pH 5.5. Since peroxidase activity was virtually absent in the preparations from leukemic mice, the pellet fraction from homogenates equivalent to those of cells from normal animals that contained 0.03 guaiacol unit was used in the experiment. Incubation was for 15 min at 37°. Dilutions and plating of bacteria were similar to that used with intact spleen cells except that the initial dilution with saponin was not required.

Glucose oxidation was determined in single side-arm Warburg flasks using intact spleen cell suspensions and either glucose-1-14C or -6-14C as the substrate. The center well of each flask contained 0.2 ml 20% KOH for trapping the CO2-14C. Reactions were terminated after 60 min incubation at 37° by tipping in 0.2 ml 30% trichloroacetic acid from the side arm. The main compartment of each flask contained 2 X 107 spleen cells, 15 μmoles glucose-14C (specific activity 0.01 μCi/μmole), and Krebs-Ringer phosphate buffer, pH 7.4, in a volume of 3.0 ml. Radioactivity of aliquots from the center well was determined in a Packard Tri-Carb liquid scintillation spectrometer equipped with an external standard for quench correction.

G6PDH activity was determined by the change in absorbance of NADP at 340 nm. Intact spleen cells suspended in 70% glycerin served as the source of enzyme (15, 20). Peroxidase activity was determined by the guaiacol procedure using the 20,000 × g pellet fraction of spleen cell homogenates as the source enzyme (23). Peroxidase-mediated amino acid oxidation used the same cell fraction as the source of enzyme and was done by previously published procedures (21, 23). The complete system in a single side-arm Warburg flask contained 5.4 μmoles L-alanine-1-14C (specific activity 0.01 μCi/μmole), 20,000 × g pellet fraction of spleen cell homogenate containing 0.03 guaiacol unit of peroxidase activity, 2 X 10-4 M H2O2, and Krebs-Ringer phosphate buffer (pH 5.5), to 3.0 ml in the main compartment. The center well contained 0.2 ml of 20% KOH for trapping CO2-14C. The reactions were run for 60 min at 37° and were stopped by addition of 0.2 ml of 30% trichloroacetic acid from the side arm. Radioactivity of the center well contents was determined as noted above. Control flasks for amino acid-14C, peroxidase, and H2O2 were run simultaneously with the complete system. When spleen cells from leukemic mice were used in these experiments, the 20,000 × g pellet of homogenates equivalent to the number of cells from normal animals was the source of enzyme.

Formate oxidation, a measure of H2O2 production, was determined by the production of CO2-14C from sodium formate-14C. These experiments were performed with intact spleen cells. The procedure was as previously described (13, 22).

Radioactive glucose and sodium formate were purchased from New England Nuclear, Boston, Mass. All other chemicals used in these experiments were purchased from commercial sources and were of reagent grade.

RESULTS

Spleen cell suspensions from AKR mice diagnosed as leukemic by splenomegaly and alterations in peripheral blood and spleen cell differentials were found to be devoid of bactericidal activity against E. coli. Cells from normal animals of this strain were able to kill over 95% of a test suspension of E. coli under similar experimental conditions.

These observations led us to examine some of the metabolic parameters that have been associated with the bactericidal activity of leukocytes. A comparison of these activities of spleen cell suspensions from normal and leukemic AKR mice is shown in Table 1. The data indicate that cells from leukemic mice have a small but significant decrease in glucose-1-14C oxidation, a greater decrease in G6PDH activity, and a marked decrease in peroxidase activities as determined by both guaiacol oxidation and amino acid decarboxylation. Oxidation of glucose-6-14C does not appear to be different from normal on a per cell basis, while formate-1-14C oxidation, a measure of cellular H2O2, is markedly higher in spleen cells from leukemic animals when compared to those from nonleukemic controls. Raising or lowering H2O2 concentrations does not alter peroxidase activity of spleen cell homogenates from leukemic animals. A 4-fold increase in the amount of leukemic homogenate also does not significantly change this activity.

Since the changes in peroxidase activity and cellular H2O2 were most dramatic, we next examined the peroxidase-con-

<table>
<thead>
<tr>
<th>Metabolic activity</th>
<th>Units</th>
<th>Normal</th>
<th>Leukemic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-1-14C oxidation</td>
<td>nmoles CO2-14C/10⁶ cells/hr</td>
<td>52.1 ± 2.9 (10)</td>
<td>42.3 ± 7.4 (10)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Glucose-6-14C oxidation</td>
<td>nmoles CO2-14C/10⁶ cells/hr</td>
<td>14.3 ± 1.4 (12)</td>
<td>13.1 ± 3.6 (5)</td>
<td>&gt;0.10</td>
</tr>
<tr>
<td>G6PDH</td>
<td>μmoles NADP reduced/10⁶ cells/min</td>
<td>0.27 ± 0.04 (12)</td>
<td>0.12 ± 0.02 (5)</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>Peroxidase-guaiacol</td>
<td>units/10⁶ cells (homogenate)</td>
<td>0.099 ± 0.022 (8)</td>
<td>0.004 ± 0.004 (7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Peroxidase-L-alanine decarboxylation</td>
<td>nmoles CO2-14C/3 X 10⁶ cells/hr</td>
<td>54.7 ± 4.7 (8)</td>
<td>1.5 ± 0.8 (7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Formate-1-14C oxidation</td>
<td>nmoles CO2-14C/10⁶ cells/hr</td>
<td>0.92 ± 0.14 (5)</td>
<td>8.50 ± 0.66 (3)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Mean ± S.E.; number of experiments in parentheses. Statistical significance was determined by Student's t test.

Animals used in these experiments were paired for age and sex. Leukemia was induced by injection of 5 X 10⁸ spleen cells from leukemic animals, into 6- to 12-week-old nonleukemic recipients. Spleens from these leukemic mice were utilized for the glucose-1-14C and -6-14C oxidation and peroxidase experiments. The animals used in the remaining experiments were all 10 months of age or more and leukemia, when present, was spontaneous. Please refer to text for complete experimental details.

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taining 20,000 X g pellet fractions of spleen cell homogenates from normal and leukemic mice for bactericidal activity. The pellet fractions from the spleens of nonleukemic mice were adjusted to contain 0.03 guaiacol unit of peroxidase activity. Because spleen cells from leukemic mice were essentially devoid of this enzymatic activity, the 20,000 X g pellet fractions from leukemic spleen homogenates were obtained from a number of cells equivalent to that containing 0.03 guaiacol unit in normal animals. The results in Table 2 show that the 20,000 X g pellet fraction of spleen homogenates from leukemic mice have no demonstrable bactericidal activity. As expected, the same fractions of spleen homogenates from normal animals are quite potent against E. coli under the same experimental conditions.

The results at this time seemed to indicate that the lack of bactericidal activity of spleen cells from leukemic mice may be related to the observed marked deficiency in peroxidase activity. In order to determine whether the decreased enzymatic activity was due to the presence of an inhibitor or to a deficiency of the enzyme per se, the 20,000 X g pellet fraction from homogenates of leukemic spleen was added to the same fraction of spleen homogenates from normal mice and L-alanine-1-14C decarboxylation in the presence of H2O2, chloride, and acidic pH was determined. The data in Table 3 show that the leukemic spleen homogenate fraction causes a significant decrease in CO2-14C production mediated by the enzyme from the spleens of normal animals. It appears from this observation that the 20,000 X g pellet fraction of spleen cell homogenates from leukemic AKR mice contains a factor that is inhibitory to the peroxidase activity found in the same fraction of splenic homogenates from nonleukemic animals of the same strain. Some preliminary experiments indicate that the inhibitory effect on peroxidase-mediated amino acid decarboxylation is stable to 100° for 30 min and is not diluted out by dialysis. The results shown in Chart 1 reveal that the degree of inhibition of peroxidase-mediated amino acid decarboxylation is directly related to the amount of the inhibitory fractions from leukemic homogenates added to the reaction mixture. As shown in this chart, doubling the amount of nonleukemic fraction is not inhibitory to the reaction. Substitution of bovine serum albumin (1.0 and 5.0 mg) for the

Table 2

<table>
<thead>
<tr>
<th>Bactericidal activity of peroxidase-containing fractions of spleen cell homogenates from normal and leukemic AKR mice</th>
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</thead>
<tbody>
<tr>
<td>E. coli control</td>
</tr>
<tr>
<td>H2O2 control</td>
</tr>
<tr>
<td>20,000 X g normal pellet control</td>
</tr>
<tr>
<td>20,000 X g leukemic pellet control</td>
</tr>
<tr>
<td>Normal pellet and H2O2</td>
</tr>
<tr>
<td>Leukemic pellet and H2O2</td>
</tr>
</tbody>
</table>

The above data are averages from 2 experiments involving 2 normal spleen preparations and 5 leukemic spleen homogenates. Each tube contained 1 x 10⁶ E. coli per ml, and where indicated 5 x 10⁻⁵ M H2O2, 20,000 x g pellet from homogenate of 2.8 x 10⁷ cells containing 0.03 guaiacol unit of peroxidase activity or, in the case of the preparation from leukemic animals, homogenates from an equivalent number of cells, and Krebs-Ringer phosphate buffer, pH 5.5, in a volume of 6.0 ml.

Table 3

<table>
<thead>
<tr>
<th>Effect of addition of 20,000 X g pellet from leukemic spleen homogenate on L-alanine-1-14C decarboxylation by this fraction from spleen cells on nonleukemic AKR mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO2-14C (nmol)</td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Leukemic</td>
</tr>
<tr>
<td>Normal + leukemic</td>
</tr>
</tbody>
</table>

| a | Mean ± S.E.; number of experiments in parentheses. |
| b | Fraction from normal mouse spleen containing 0.03 guaiacol unit of peroxidase. |
| c | Fraction from leukemic mouse spleen adjusted to the same number of cells as present in normal. |

The data presented in this report indicate that spleen cell suspensions from leukemic AKR mice are deficient in several
functions. These include bactericidal, G6PDH, and SPO activities. The increased formate oxidation by spleen cells from leukemic mice may be related to their decreased SPO activity. A relationship between the above-mentioned enzyme activities and bactericidal activity has previously been reported to exist in spleen cells from nonleukemic mice as well as in phagocytic cells from a variety of animals and man (8, 16, 18).

It has been reported that leukocytes deficient in G6PDH and hexose monophosphate shunt activities due to genetic defects (2), phenylbutazone (9, 24), and acute leukemia (19, 20) also have a defect in bactericidal activity. Hence the effects in bactericidal and associated metabolic functions observed in spleen cell suspensions from leukemic AKR mice seem to parallel those observed in leukocytes from patients with genetic deficiency of G6PDH, animals with chemically blocked activity of this enzyme, and patients with acute leukemia.

The relationship of peroxidase activity to bactericidal function of leukocytes is more direct than that of G6PDH activity. It has been shown that the in vitro reaction of peroxidase with sublethal concentrations of H₂O₂ in the presence of chloride, iodide, or bromide at acidic pH is lethal to a variety of microorganisms (6). These include bacteria, both Gram-positive and Gram-negative varieties, yeasts, fungi, viruses, and Mycoplasma (1, 5, 6, 10). Peroxidases effective in this system have been obtained from guinea pig peritoneal exudate polymorphonuclear leukocytes, guinea pig bone marrow leukocytes, human peripheral blood leukocytes, rabbit alveolar macrophages, and mouse spleen cell suspensions (6, 8, 14, 16—18, 22).

When the reaction is run in the presence of chloride it has been postulated that aldehyde formation due to peroxidase-mediated deamination and decarboxylation of microbial amino acids is responsible for the observed antimicrobial activity (18).

Since spleen cell suspensions from leukemic AKR mice exhibit marked deficiencies in both peroxidase and bactericidal activities, one could postulate that the peroxidase-H₂O₂-chloride system plays a major role in the antimicrobial function of these cells. The observed inhibitory effect of the

<table>
<thead>
<tr>
<th>Source of fraction</th>
<th>E. coli/ml after 15 min at 37°</th>
<th>% bactericidal activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial controls</td>
<td>4.3 x 10⁶</td>
<td></td>
</tr>
<tr>
<td>Normal mouse spleen</td>
<td>2.1 x 10⁷</td>
<td>99.93</td>
</tr>
<tr>
<td>Leukemic mouse spleen</td>
<td>3.6 x 10⁷</td>
<td>18.00</td>
</tr>
<tr>
<td>Normal mouse spleen and leukemic mouse spleen</td>
<td>4.0 x 10⁷</td>
<td></td>
</tr>
</tbody>
</table>

The data in Tables 3 and 4 as well as those in Chart I indicate that the inhibitory effects are too great to be caused by simple dilution. The data appear to indicate that the 20,000 X g fraction 2-fold does not result in an inhibitory effect would appear to eliminate the involvement of excess proteins or amino acids as the inhibitor. This observation also indicates that the normal 20,000 X g fraction used in these experiments contained nonsaturating levels of SPO. Hence, the observed inhibitory effects of the leukemic 20,000 X g fraction are minimal with respect to amino acid decarboxylation. The observed complete inhibition of bactericidal activity may be due to the composition of the system involved. The peroxidase-mediated bactericidal reaction contains only 5 X 10⁻⁵ M H₂O₂, whereas the amino acid decarboxylation reaction uses 2 X 10⁻⁴ M H₂O₂. This 4-fold difference in H₂O₂ concentration is probably significant with respect to the degree of inhibition of the reaction. It thus becomes obvious that isolation and characterization of this inhibitory factor will be of considerable interest.

Patients with acute leukemia exhibit increased susceptibility to infectious diseases (4, 11, 25). Peroxidase activity of leukocytes from leukemic patients has not yet been extensively studied. However, there is a report that leukocytes from a patient with myelomonocytic leukemia have decreased peroxidase activity, and there is another report of increased serum peroxidase activity in patients with leukemia (3, 12). It is possible that a factor inhibitory for the peroxidase-H₂O₂-chloride bactericidal system similar to that reported here for leukemic mice may also be present in leukocytes from patients with some forms of leukemia. The peroxidase-mediated antimicrobial activity of leukocytes from leukemic patients remains to be evaluated. From the data presented in this report and elsewhere, it seems that in-depth studies of this activity in leukemic leukocytes might further our knowledge of why leukemia patients become prone to bacterial infections.

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