The Inhibitory Nature of Lymphocytic Leukemic Serum on Microorganisms*

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SUMMARY

The sera of patients afflicted with either acute or chronic lymphocytic leukemia were found to significantly inhibit the respiratory activity of Bacillus subtilis to a much greater extent than any other sera tested. The inhibitor is destroyed by heating at 65° C. for 30 min., it can be absorbed out by heat-killed homologous organisms, and is nondialyzable. These data indicate that the factor is probably a protein. The inhibitor appears to cause bacteriostasis, since the addition of either yeast extract or trypticase soy will reverse the inhibitory effect brought about by the acute or chronic lymphocytic leukemic serum.

In a study designed to explore the opsonic activity of leukemic serum it was found that the serum of patients with acute or chronic lymphocytic leukemia inhibited the respiratory activity of different microorganisms. Some inhibition was also noted in the serum from patients with Hodgkin's disease and lymphosarcoma. Normal serum, chronic myelocytic and monocytic leukemia, multiple myeloma, di Guglielmo's syndrome, and hemolytic anemia all behaved similarly (i.e., the organism tested exhibited a relatively consistent high respiratory activity in their presence).

The finding of serum-inhibitors (bactericidins) dates back to the early days of microbiology (see review of Skarnes and Watson [4]). However, it appears that specific studies on the inhibitory activity of sera from patients afflicted with leukemia have not been reported. In the present study sera obtained from patients with either acute or chronic lymphocytic leukemia (ALL or CLL) have been found to inhibit the respiratory activity of Bacillus subtilis and, to a lesser extent, Micrococcus pyogenes var albus, M. pyogenes var aureus, and some strains of Escherichia coli. The inhibitory factor was found to be most active against gram-positive organisms.

It is the purpose of this paper to report on the presence and nature of a substance(s) found in the serum of patients with acute and/or chronic lymphocytic leukemia that will inhibit the respiratory activity of different microorganisms.

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MATERIALS AND METHODS

Organisms.—The strains of Bacillus subtilis, Micrococcus pyogenes var albus, M. pyogenes var aureus, and Escherichia coli were obtained from the stock culture collection of the Department of Pathology and Medical Research of St. Margaret's Hospital.

Plating media.—The minimal medium was that used by Stapleton, Sbarra, and Hollaender (5). Trypticase soy broth (BBL) or yeast extract (Difco), 30 mg. per plate, was added to the minimal medium, when desired.

Respiratory measurements were made by use of conventional Warburg manometry (6). The system consisted of 0.5 ml. of an 18-hour culture of B. subtilis (equivalent to 5 x 10⁶ cells) grown in trypticase soy broth and washed twice with M/15 phosphate buffer of pH 7.4. The cells were placed in the main compartment of Warburg flasks along with 1 ml. of either trypticase soy broth (30 mg.), yeast extract (3 mg.), or M/15 phosphate buffer. The total volume in this compartment was brought to 2.4 ml. with M/15 phosphate buffer; 0.6 ml. of test serum was added from the sidearm after an appropriate equilibration period. The center well contained 0.2 ml. of a 20 per cent KOH solution. All the experiments were done at 37° C.

Plate counts, when desired, were made in trypticase soy or yeast extract or minimal agar by conventional methods.

Sera.—Normal sera were obtained from male and female hospital personnel from 19 to 65 years old. In total, 44 patients, varying in age from 17 to 74 years, were employed. The leukemic patients were known to have had Sera from patients with leukemia and other diseased states were obtained through the kindness of Dr. William Dameshek of the Blood Research Laboratory of the New England Center Hospital, Dr. William Maloney of the Hematology Laboratory of the Boston City Hospital, Dr. Willy Pachas of the Lemuel Shattuck Hospital, and Dr. Bernard Maney of St. Margaret's Hospital.
their disease for a minimum of 6 months. The state of the disease, the presence of infection, the febrile status, and the age or sex of the patient did not appear to influence the inhibitory activity. Thus, the sera of patients with all stages of disease showed the same activity. Sera from patients under treatment were not used in this study. Approximately 10 ml. of blood was drawn from all subjects by aseptic technic, allowed to clot at room temperature for 1 hour, and then separated by centrifugation. The serum obtained (free of hemolysis) was then frozen and used when desired.

Absorption of serum.—Serum was absorbed with a heat-killed, twice-washed, 24-hour culture of *B. subtilis*. The absorption was carried out in the cold room (4°C) overnight. The reaction mixture contained: 1.0 ml. serum, 1.0 ml. packed cells, and 1.0 ml. m/15 phosphate buffer. After incubation in the cold this mixture was centrifuged at 5000 × g for 15 minutes, and the supernatant was tested immediately.

*Serum dialysis.*—Dialysis of serum was carried out overnight in the cold (4°C.) against physiological saline. The saline was changed at 3-hour intervals over a 9-hour period.

**RESULTS**

The effect of normal serum and serum from a patient afflicted with chronic lymphocytic leukemia (CLL) on the respiratory activity of *Bacillus subtilis* is shown in Chart 1. The respiratory activity of this organism in the presence of CLL serum was significantly less compared with its respiratory activity in the presence of normal serum. The concentration of serum shown in this experiment was 20 per cent, and the inhibitor could be titrated and was without effect when the concentration was reduced to 2 per cent. Other microorganisms were also titrated and was without effect when the concentration was reduced to 2 per cent. Other microorganisms were also tested and found to be sensitive to the serum factor from CLL and/or ALL patients. Since the variations between sera from CLL and ALL patients were identical, the results of both groups were combined. Some strains of *E. coli* and *M. pyogenes*, both the *albus* and *aureus* varieties, also showed marked respiratory inhibition; however, others were resistant to the effects of the serum inhibitor. Since the inhibition with *B. subtilis* was pronounced and consistent, this organism was used exclusively in the studies reported in this paper.

The effects of sera from patients afflicted with various diseases on the respiratory activity of *B. subtilis* are shown in Table 1. This survey of sera revealed that the inhibitory activity is found in patients having CLL and to a somewhat lesser extent in patients having Hodgkin’s disease and lymphosarcoma. The greatest inhibitory activity was observed with serum from CLL or ALL patients.

<table>
<thead>
<tr>
<th>Temperature*</th>
<th>Normal serum</th>
<th>CLL serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>100†</td>
<td>16</td>
</tr>
<tr>
<td>56°C</td>
<td>84</td>
<td>12</td>
</tr>
<tr>
<td>65°C</td>
<td>316</td>
<td>340</td>
</tr>
</tbody>
</table>

* Sera held at designated temperatures for a 30-min. incubation period before assay for inhibitory activity.

† µl. oxygen uptake at 3 hours set at 100 per cent. Each result is a mean from three different experiments.

Final serum concentration in all cases, 20 per cent.
sera of patients with CLL diseases is similar to inactivation of other proteins by heat.

Incubation of sera from CLL patients with heat-killed *B. subtilis* cells overnight at 4° C. resulted in the removal of the inhibitor substance (Table 3). It should be noted that inhibitor substance is removed from both normal and CLL serum. The serum could be kept in the frozen state for at least 1 month without loss of activity, even when repeatedly thawed and refrozen. Furthermore, the material was found to be nondialyzable (Table 3). The above data obtained from typical experiments, and the activity of the material after dialysis, give further evidence that the substance is protein in nature and that the protein is not associated with small molecular weight co-factors.

Cells exposed to CLL serum for 2 hours were unable to grow well on minimal medium. The inhibitor appears to cause bacteriostasis, since the addition of either yeast extract or trypticase soy to the minimal medium reversed the inhibitory effect completely (Table 4). Cells treated with normal serum also exhibited a bacteriostatic effect (20 per cent) which was relieved by the addition of yeast extract.

**TABLE 3**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Oxygen uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>100*</td>
</tr>
<tr>
<td>CLL</td>
<td>30</td>
</tr>
<tr>
<td>Absorbed normal†</td>
<td>164</td>
</tr>
<tr>
<td>Absorbed CLL†</td>
<td>138</td>
</tr>
<tr>
<td>Dialysed normal‡</td>
<td>107</td>
</tr>
<tr>
<td>Dialysed CLL‡</td>
<td>38</td>
</tr>
</tbody>
</table>

* ‡ DIALYSIS CARRIED OUT AT 4° C. OVERNIGHT AGAINST REPEATED CHANGES OF 0.9 PER CENT NaCl.
† DIALYSIS CARRIED OUT AT 4° C. OVERNIGHT AGAINST REPEATED CHANGES OF 0.9 PER CENT NaCl. Final serum concentration in all cases 20 per cent.

**TABLE 4**

<table>
<thead>
<tr>
<th>Bacteria exposed to,*</th>
<th>Plated on</th>
<th>Minimal medium†</th>
<th>Minimal medium plus yeast extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heated normal or heated CLL serum (65° C./30 min.)</td>
<td>100‡</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Normal serum</td>
<td>80</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>CLL serum</td>
<td>47</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

* CELLS EXPOSED TO DESIGNATED SERUM AT 37° C. FOR 120 MINUTES. ‡ VIABLE COUNT SET AT 100%. SEE TEXT FOR ADDITIONAL DETAILS.
† SUBSEQUENTLY PLATED ON MINIMAL AND MINIMAL PLUS 30 MG. YEAST EXTRACT. INCUBATED OVERNIGHT AT 37° C.
‡ VIABLE COUNT SET AT 100%. SEE TEXT FOR ADDITIONAL DETAILS.

Either yeast extract or trypticase soy also relieved the inhibition of respiration observed with CLL serum (Chart 2).

**DISCUSSION**

The capacity of serum to inhibit the growth of microorganisms has been known and has interested microbiologists ever since the latter part of the 19th century (4). However, the possible significance of these factors in vivo has been and remains debatable. A factor(s) present in the serum of patients afflicted with CLL or ALL has been found which inhibits significantly the respiratory activity of some gram-positive organisms—in particular, *B. subtilis*. Normal serum also has the capacity to inhibit the respiratory activity of this organism but to a much lesser extent than CLL serum. Some inhibition was also observed with serum from patients with lymphosarcoma and Hodgkin's disease; this is of interest, since both of these diseases are of lymph node origin and are thus related to CLL. All other sera tested react in a manner similar to that of normal serum (Table 1).

It is generally accepted that respiratory activity parallels viability. That the factor in CLL or ALL affects bacterial viability is borne out by the inhibition of the growth of *B. subtilis* in minimal medium as well as by its effect on respiration. The reversal of this inhibition of respiration by yeast extract or trypticase soy and the reversal of minimal media of bacteriostasis by these nutrients show that the two substances counteract the effect of the factor and may be related. This work is being continued, since it is of interest to pursue the nature of the compound(s) in yeast extract or trypticase soy responsible for the reversal so that some insight into the mechanism may be provided. This reversal appears to be similar to that observed frequently in bacteria exposed to deleterious agents. For example, Stapleton, Sbarra, and Hollaender...
(5) have shown that *E. coli* B/r will partially recover from an exposure to either x- or γ- radiation by plating after exposure on a minimal medium containing yeast extract.

Many reports (i.e., Jacox [1], Myrvik [2]) have appeared in the literature indicating an enhanced bactericidal activity of human serum for gram-positive organisms from patients suffering with different illnesses—e.g., carcinoma, virus, and bacterial infections and during acute coronary occlusion. To date we have not been able to show the presence of the characteristic activity (level of inhibition) of this factor in any other condition tested other than the ALL and/or CLL and CLL-related disease states (Table 1). This factor may be peculiar to the lymphocytic leukemias and consequently may be associated with the lymphocytic cells. Thus, an attempt to show a correlation between the lymphocytes and the factor is now in progress.

The fact that bacterial infections are common in patients suffering from CLL would indicate that the significance of the factor is at best questionable. However, many of the infections reported in these patients have been attributed to the concomitant administration of adrenal corticosteroids (3). In a subsequent publication it will be shown that certain forms of therapy interfere with the inhibitory activity of the factor (E. Ouchi and A. J. Sbarra, The Effect of Steroids and Leukemic Serum on the Respiratory Activity of *Bacillus subtilis*. *Nature* (in press).

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