Detection of Endotoxin in Commercial L-Asparaginase Preparations by Complement Fixation and Separation by Chromatography

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SUMMARY

Of 15 L-asparaginase preparations tested, 13 inhibited whole human serum complement; the inhibitory effect ranged from 12 to 45%. We have partially characterized the anticomplementary activity in one of the L-asparaginase preparations. The anticomplementary activity was separable from the L-asparaginase activity on Sephadex G-200. Fractions containing L-asparaginase had no effect either on whole human serum complement or on human serum Cl, the first component of complement; these fractions were therapeutic in leukemic mice. Fractions containing the anticomplementary activity had no significant therapeutic effect in leukemic mice. L-asparaginase and the anticomplementary factor are antigenically distinct, and the anticomplementary factor was shown to be antigenically related to Escherichia coli lipopolysaccharide (endotoxin).

INTRODUCTION

A-Ase, first isolated from guinea pig serum, inhibits tumors the growth of which depends upon the presence of L-asparagine (2, 6). The therapy of such diseases with A-Ase isolated from E. coli is often associated with side effects like renal failure, pancreatitis, abnormalities of clotting factor, and edema (5, 10). It was recently reported that A-Ase (E. coli) in vitro inhibits human and guinea pig whole serum complement and that A-Ase is a potent activator of Cl (4). It was therefore proposed that these results may give the explanation for the severe anaphylactic reactions noted in some patients who received the drug. Because guinea pig serum contains A-Ase in high quantities and because the authors' data were obtained with reagents and methods that made their data difficult to interpret on a molecular basis, we have reinvestigated the effect of A-Ase (E. coli) preparations on human and guinea pig complement.

In a 1st report (7) we could show: (a) that neither of the A-Ase preparations tested destroyed guinea pig complement activity; (b) that of the 2 lots of A-Ase, one fixed only a small amount of human complement in human sera while the other fixed all measurable complement in human sera; (c) that activation of Cl by a given lot of A-Ase depends on the source of human serum; and (d) that a small but significant reduction in late complement component activity could be measured in human sera exposed to A-Ase preparations.

These results supported the hypothesis that the effects of A-Ase preparations on human complement are due to a contaminant that is present in different quantities in different lots of A-Ase.

In this report we present the following evidence for the above hypothesis: A-Ase by itself has no effect on human complement; some A-Ase preparations contain a contaminant acting on human complement which is separable from the enzyme activity; this contaminant has no effect on mouse leukemias and is immunologically different from A-Ase.

MATERIALS AND METHODS

A-Ase (NSC 109229, Merck, Sharp and Dohme, Rahway, N.J.), cyclophosphamide (NSC 26271), and methotrexate (NSC 740) were supplied by Drug Research and Development, Chemotherapy, National Cancer Institute, Bethesda, Md. A-Ase was constituted in 0.9% NaCl solution, and the designated doses were expressed as units/ml determined by the nesslerization method as described in Ref. 12. Serum samples from 2 individual humans (ML and FS sera) served as the source of complement. Reagents and methods used for the determination of whole serum complement (CH50 units) and for Cl are described in Refs. 8 and 11.

Male Hartley guinea pigs weighing more than 500 g were obtained from stock maintained at the NIH, Bethesda, Md. Groups of guinea pigs were immunized with either A-Ase (100 μg; Lot 8067) or pools of Sephadex G-200 (Pharmacia, Uppsala, Sweden) gel chromatography fractions of the same A-Ase preparation. These materials were emulsified with equal volumes of complete Freund's adjuvant (H37 Ra, Difco Laboratories, Detroit, Mich.) and injected in 0.1-ml aliquots into each of the 4 footpads. The animals were skin tested with

Received May 31, 1972; accepted July 12, 1972.

1 Guest worker in the Biology Branch, National Cancer Institute. Supported by the Deutsche Forschungsgemeinschaft Lo 188/1.
2 To whom reprint requests should be sent, at National Cancer Institute, Building 37, Room 2B15, Bethesda, Md. 20014.
3 The abbreviations used are: A-Ase, L-asparaginase; Cl, the 1st component of complement; MST, median survival time; ILS, % increase in MST.
10 μg of each of the substances used for immunization 12 days after initial immunization. On Day 21, each animal was given an intradermal injection of 10 μg of *E. coli* 026:B6 lipopolysaccharide W (3121-25, Difco Laboratories). Skin reactions were observed 6, 24, and 48 hr after injection. There was no immediate reaction. The maximal skin reaction (24 hr) was estimated by measuring 2 perpendicular diameters of each lesion. The results of each group of animals are expressed as the average radii squared of reactions ± S.E.

Ten- to 12-week-old BALB/c × DBA/2 F₁ mice (hereafter called CDF₁) were inoculated with leukemia L5178Y ascites cells that had been maintained in DBA/2 mice. The cell counts in the ascites tumor cell suspensions were determined with the aid of a hemocytometer, and the cell concentration of the inoculum was adjusted and inoculated s.c. (10⁶ cells/0.25 ml) into the right axillary area of CDF₁ mice. At the time of treatment A-Ase and A-Ase fractions were constituted in 0.9% NaCl solution, and the designated doses were prepared in 0.2-ml volumes and injected i.p. Cyclophosphamide was dissolved in 0.9% NaCl solution, and methotrexate was dissolved in 2% sodium bicarbonate solution. The drugs were injected i.p. in a constant volume of 0.01 ml/g body weight at doses as shown in the tables and charts. Body weights and tumor diameters of the mice were taken at regular intervals.

**RESULTS**

**Effect of A-Ase Preparations on Whole Serum Complement.**

In an earlier report we found that 2 different lots of A-Ase fixed different amounts of human complement (7). We now tested the effect of 15 different lots of A-Ase on human serum complement. Equal volumes of undiluted serum (ML serum) and A-Ase (containing 500 units/ml) were mixed and incubated at 37° for 1 hr. At this time the samples were diluted and the amount of hemolytic complement was quantitatively determined. The results (Table 1) show that only 2 A-Ase preparations (Lots 7633 and 7938) were without any effect on whole complement. The inhibitory effect of the other preparations ranged from 12 to 45%. Since the amount of A-Ase was the same in all preparations exposed to human serum, these data support the hypothesis that the effect of the A-Ase preparations on complement is due to a contaminant that is present in different quantities in different lots of A-Ase (7).

**Separation of the Anticomplementary Activity from the A-Ase Activity.** To test this hypothesis we subjected A-Ase preparation Lot 8067 to molecular sieve chromatography on Sephadex G-200 (elution buffer, 0.15 M NaCl) (1). The effluent was tested for A-Ase activity and for interaction with complement. The results shown in Chart 1 indicate that the 2 activities are separable. The anticomplementary activity appeared in the void volume while the A-Ase activity was included by the beads, and its position with respect to serum proteins was between the 7S and albumin peak. In our earlier paper we reported that incubation of an unfractionated A-Ase preparation with some human sera led to activation of C₁ (7). The fractions which were found to be anticomplementary to whole human serum were also responsible for action on serum C₁ (Chart 2). Fractions containing A-Ase activity had no effect on whole human serum complement or on C₁ in human serum.

**The Effect of the Anticomplementary Factor and Purified A-Ase on Leukemic Mice.** To see whether the anticomplementary factor separable from A-Ase activity has an effect on leukemic mice, we performed a similar fractionation of Lot 8067 on Sephadex G-200 as described above. From the fractions 3 pools were formed: Pool 1, containing the
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anticomplementary activity; Pool 2, containing fractions between the peak of the anticomplementary activity and the peak of A-Ase activity; and Pool 3, containing the A-Ase activity. The effect of a single treatment with A-Ase or A-Ase fractions on the survival time of leukemic mice is shown in Table 2.

The MST of untreated control mice inoculated with $10^6$ cells s.c. was 14.5 days. The MST of mice treated on Day 3 with 9 to 180 units of unfractionated A-Ase varied from 20.5 to 31.5 days; ILS over untreated controls ranged from 41 to 117%. Treatment with Pool 1 was not effective; the ILS of mice treated with Pool 1 was only 3 to 17% over the untreated controls. A moderate increase in survival time of 16 to 21 days (ILS, 10 to 45%) was obtained in mice treated with Pool 2, whereas Pool 3 had similar effect as A-Ase on survival time of leukemic mice (MST, 19.5 to 29.5 days; ILS, 34 to 103%).

With a single treatment with cyclophosphamide, 200 mg/kg, the MST was 26.5 days (ILS, 83%), and daily treatment with methotrexate (3 mg/kg, Days 3 to 7) increased the MST to 18.5 days (ILS, 28%). Significant body weight losses were not observed during the 1st 10 days in all the experimental animals.

The average tumor diameter was 8 mm by Day 7 in the untreated controls and reached a size of 16 mm on Day 13. In the mice treated with Pool 1 and Pool 2, tumor diameters were about 5 to 8 mm on Day 7, and by Day 17 they grew to a size of 14 to 17 mm as in the untreated controls. However, tumor growth was delayed in the mice treated with the

![Chart 2. Gel filtration of A-Ase preparation on Sephadex G-200 in 0.15 M NaCl. o, A-Ase activity; o, anticomplementary activity; e, C1 in FS serum after treatment with the fractions; o, C1 in ML serum after treatment with the fractions. C, complement; C1, activated form of C1.](chart2.png)

Table 2

Comparison of therapeutic effectiveness of several fractions of A-Ase Lot 8067 in leukemic (L5178Y) mice

CDF, mice were inoculated s.c. with $10^6$ L5178Y ascites cells; drug administration took place 3 days after tumor inoculation.

<table>
<thead>
<tr>
<th>A-Ase Lot 8067</th>
<th>Pool 1$^b$</th>
<th>Pool 2$^b$</th>
<th>Pool 3$^b$ 200 units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-Ase units i.p.</td>
<td>MST$^a$ (days)</td>
<td>Range (days)</td>
<td>ILS over untreated controls</td>
</tr>
<tr>
<td>180</td>
<td>31.5</td>
<td>28–34</td>
<td>117</td>
</tr>
<tr>
<td>65</td>
<td>28.0</td>
<td>23–31</td>
<td>93</td>
</tr>
<tr>
<td>39</td>
<td>26.5</td>
<td>22–30</td>
<td>83</td>
</tr>
<tr>
<td>23</td>
<td>24.5</td>
<td>13–27</td>
<td>69</td>
</tr>
<tr>
<td>14</td>
<td>23.5</td>
<td>21–27</td>
<td>62</td>
</tr>
<tr>
<td>9</td>
<td>20.5</td>
<td>18–22</td>
<td>41</td>
</tr>
</tbody>
</table>

Uninvolved tumor control

| 10$^6$ cells | 14.5 | 13–18 | 0 |

Cyclophosphamide, 200 mg/kg

| 26.5 | 16–34 | 83 |

Methotrexate, 3.0 mg/kg

| 18.5 | 15–21 | 28 |

$^a$ Of 20 untreated controls, 8 mice in treated groups.

$^b$ Pool 3, containing 200 units A-Ase per ml, was diluted with 0.9% NaCl solution to the same amount of A-Ase activity as used in the unfractionated A-Ase preparation; Pools 1 and 2 were diluted as Pool 3. A-Ase preparation was fractionated on Sephadex G-200. Pools were made as described in text.
Endotoxin in Asparaginase (E. coli) Preparations

Table 3

<table>
<thead>
<tr>
<th>Immunization with</th>
<th>Lot 8067, 10 μg</th>
<th>Pool 1, 10 μg</th>
<th>Pool 3, 10 μg</th>
<th>E. coli 026:B6 Lipopolysaccharide, 10 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot 8067, 100 μg</td>
<td>68.2 ± 4.1</td>
<td>19.6 ± 5.5</td>
<td>68.1 ± 2.4</td>
<td>15.4 ± 0.6</td>
</tr>
<tr>
<td>Pool 1, 24 μg</td>
<td>2.9 ± 1.1</td>
<td>26.8 ± 2.3</td>
<td>1.6 ± 0.4</td>
<td>24.7 ± 4.9</td>
</tr>
<tr>
<td>Pool 3, 100 μg</td>
<td>52.1 ± 16.1</td>
<td>7.1 ± 2.0</td>
<td>81.6 ± 13.5</td>
<td>5.0 ± 2.7</td>
</tr>
<tr>
<td>Nonimmunized</td>
<td>3.1</td>
<td>4.0</td>
<td>1.0</td>
<td>3.1</td>
</tr>
</tbody>
</table>

24-hr skin reaction (av. sq radii in sq mm ± S.E.) to

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<thead>
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<th>lot 8067, 10 μg</th>
<th>Pool 1, 10 μg</th>
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**DISCUSSION**

Delage *et al.* (4) recently reported that A-Ase (E. coli) in *vitro* inhibits human and guinea pig whole serum complement and that A-Ase is a potent activator of C1. These authors therefore proposed that these results may give the explanation for the severe anaphylactic reactions noted in some patients on administration of the drug. Reinvestigating these experiments, we showed that the effect of A-Ase preparation on human complement or on human C1 was not an effect of the A-Ase activity (7). We therefore proposed that the anticomplementary effect of A-Ase preparation was due to the presence of bacterial contaminants in different quantities in different lots of A-Ase. Testing 15 different lots of A-Ase (Table 1), we found only 2 lots without any effect on human complement; the anticomplementary effect of the 13 other A-Ase preparations range from 12 to 45%. The anticomplementary activity was separable from the A-Ase activity by gel chromatography on Sephadex G-200 as shown in Chart 1. Fractions containing A-Ase activity had no effect either on whole serum complement or on human serum C1. Fractions containing the activity acting on whole serum complement also activated human C1 in FS serum, while they did not act on human C1 in ML serum (Chart 2). This is in agreement with our earlier finding that activation of C1 by a given lot of A-Ase depends on the source of human serum (7). The anticomplementary activity separated from the A-Ase activity has no therapeutic effectiveness on the survival time of leukemic mice, while the purified A-Ase showed the same effect on leukemic mice as the unfractionated A-Ase preparation.

The anticomplementary contaminant did not show an immunological cross-reaction with A-Ase but did cross-react with *E. coli* lipopolysaccharide (endotoxin). This finding is strong evidence that the contaminant of the A-Ase preparation is endotoxin. This is further supported by the Limulus assay for endotoxin (3), which was positive in those A-Ase preparations that were anticomplementary to whole human serum and negative in those preparations that were not anticomplementary (M. Loos and T. Borsos, unpublished observations). Since many human sera contain natural antibodies to *E. coli* antigens (most likely endotoxin), the consumption of complement after incubation of human sera with A-Ase preparation (containing endotoxin) is a result of the formation of antigen-antibody complexes. On the other hand, it is known that the action of bacterial endotoxins on late-acting components leads to the generation of anaphylatoxin (9); we therefore propose that the immediate, anaphylactic type reactions in patients given injections of A-Ase preparation derived from *E. coli* are due to endotoxin. This interpretation is supported by the comparison of the pharmacological effects of *E. coli* A-Ase (containing endotoxin) and *E. coli* endotoxin in which it was shown that both have similar effects in *vivo* (E. H. Herrman, Microbiological Associates, Inc., Bethesda, Md., personal communication). We therefore suggest that complement-fixing ability of A-Ase preparations could be used for screening of A-Ase lots that could be administered to patients without evoking anaphylactic reactions. Furthermore, complement fixation tests of A-Ase lots would be useful in obtaining highly pure A-Ase preparations free of bacterial contaminants.

**REFERENCES**


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