The Use of the Proteolytic Enzyme Brinase to Produce Autocytotoxicity in Patients with Acute Leukemia and Its Possible Role in Immunotherapy

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SUMMARY

Daily infusion of brinase (Protease 1 of *Aspergillus oryzae*) for 10 to 30 days in six patients (three children, three adults) with acute leukemia resulted in the production of complement-dependent autocytotoxicity against leukemic cells and lymphocytes and, in some instances, against platelets. This appeared to be due to the production of autoantibody, but its nature remains to be elucidated. The autocytotoxicity can be demonstrated *in vivo* by blood transfusion from a healthy donor or *in vitro* at 37°C by Terasaki's microcytotoxicity test. The autocytotoxicity is transient, lasting from 3 to 15 days, but repeat courses of brinase, whether they are given alone or in combination with antileukemic drugs, produce further autocytotoxic "antibodies." Remission was obtained in three of five patients who were given combination therapy and in the one patient with acute myeloblastic leukemia who was treated by brinase alone.

INTRODUCTION

A proteolytic enzyme similar to plasmin has been isolated from *Aspergillus oryzae* (2) and is now called brinase (4). Like plasmin, this enzyme can kill cancer cells in culture and, when given i.v., it lowers antiplasmin levels. In patients with acute leukemia, serum antiplasmin levels are abnormally high; the reduction of these antiplasmin levels to below 50% of normal values rapidly decreases the number of circulating leukemic cells (18).

While investigating the therapeutic effects of brinase in 25 cases of leukemia complement-dependent autocytotoxicity against leukemic cells, lymphocytes and platelets were observed in 4 patients (19). This autocytotoxicity was first demonstrated *in vivo* by a dramatic fall in white cell and platelet counts when whole-blood transfusion was given at the end of an 18-day course of brinase. The 22-year-old patient had acute lymphoblastic leukemia (20). Warfarin sodium was given as maintenance therapy to inhibit malignant cell locomotion and growth (21).

The production of autocytotoxicity by brinase was investigated further in 3 patients and is described below. It was necessary to know whether brinase could be combined with conventional antileukemic therapy or whether it could be of value if given after the patient became resistant to conventional therapy.

The ability to reproduce autocytotoxicity was the main purpose of this study, and this was accomplished on each of the 5 occasions on which it was attempted in 3 patients.

MATERIALS AND METHODS

**Brinase.** Protease 1 of *Aspergillus oryzae* is produced by Astra AB, Sweden, as a fibrinolytic agent. Astra 1652 (Lot No. 17042). Brinase was given daily by i.v. infusion for 1 hr, as previously described (20). The dosage was 2.5 mg/kg of body weight in children and 100 to 350 mg daily in adults.

Serum antiplasmin levels were used to estimate dosage, which was varied to keep antiplasmin levels between 50 and 10% of normal (100%). Full hematological analysis was performed before and after each infusion, as described (8). Bone marrow examinations were carried out weekly during therapy.

**Assay of Autocytotoxicity.** This was performed by D.J.R. at the Transplantation-Immunology Unit of Jervis Street and St. Laurence's Hospitals, Dublin, under the direction of Dr. J. G. Devlin.

We used the microdroplet method of Terasaki and McClelland (16) for the detection of lymphocytotoxic antibodies. Serum samples from patients were tested against the patient's own lymphocytes. This test consists essentially of the incubation of the patient's serum for 2 hrs at 37°C in 0.001-ml quantities with 1000 lymphocytes and 0.005 ml of complement. The killed cells were identified by eosin dye inclusion or by complete cell lysis. A test was considered negative unless more than 20% of the cells were killed. In the patients described below, all positive tests showed more than 30% killed and the negative tests showed less than 10%.

The cells from the leukemic patients were prepared by the method of Terasaki and McClelland (16). In the patients with lymphatic leukemia, all the separated cells tested were lymphocytes, but confusion arose in the case of acute myeloblastic leukemia because leukemic blast cells separated with the lymphocytes. Neither the lymphocytes nor the blast cells were killed when the patients' sera were not toxic. When the sera became cytotoxic, both lymphocytes and blast cells...
were killed. The percentage of each cell type was not calculated, but from 40 to 80% of the mixed cells were killed on each occasion of testing during the period of cytotoxicity; approximately 50% of these cells were lysed completely after 2 hr of incubation. The term "autocytotoxicity" was therefore used instead of "lymphocytotoxicity" because both lymphocytes and leukemic cells appear to be affected by positive sera.

Controls. All patients had negative tests before and after the period when autocytotoxicity was present.

We used 3 controls for each test in which 0.9% NaCl solution replaced autologous serum or complement and heat-inactivated complement replaced active complement. In addition, the patient acted as his own control because autologous serum taken when autocytotoxicity was not present did not kill with the patient's own cells or with other patients' cells.

The complement source used was nontoxic rabbit serum. Modifications of the hemolysin test (10) were used to estimate complement levels (7) and complement fixation (11).

Cross-reaction Tests. We used the autocytotoxic serum from 1 patient (Case 5) to study cross-reaction with lymphocytes from patients with other forms of cancer. In positive cross-reaction tests 30 to 50% of the lymphocytes were killed. When 10% or fewer lymphocytes were killed, the cross-reaction was considered negative. The control for these tests was serum from the same patient (Case 5) when autocytotoxicity was absent.

RESULTS

The reexamination of previous cases showed that autocytotoxicity occurred when brinase therapy was given for 7 or more days. Therefore, 3 more patients were specifically treated with brinase to induce autocytotoxicity under different conditions. All the cases are summarized in Table 1.

When the 3 patients in this study were treated with daily doses of brinase, it produced autocytotoxicity on each of 5 occasions. These are described below.

Case 4

K. M. (treated by Dr. H. E. Counihan), a 45-year-old woman with acute myeloblastic leukemia, was treated with brinase and anticoagulated with warfarin sodium. The 1st course of brinase lasted 30 days and was interrupted 1 day in 7. The daily dosage was 200 mg (total 5200 mg). Autocytotoxicity was detectable for 7 days for 30 to 40% of her leukocytes. There was a partial remission which relapsed 5 weeks later. The 2nd course of brinase was 300 mg for 4 days. After 7 weeks, a 3rd course of 300 mg was given for 5 days. Complete remission for 2 months followed, but there was no evidence of autocytotoxicity. After 10 weeks, 3 courses of 200 mg were given daily for 5 days without producing autocytotoxicity, but remission ensued for 13 months before bone marrow relapse. This condition was then treated by continuous daily infusions of 250 mg of brinase. After 10 days, autocytotoxicity developed against 75% of the patient's lymphocyte preparations (50% of these cells were completely lysed after 2 hr of incubation). Brinase was continued for another 15 days and autocytotoxicity remained throughout this time, varying between 40 and 80%. The patient was discharged in hematological remission from the hospital, and 1 week later no autocytotoxicity was detectable.

Comment. The necessity of continued daily infusions of an adequate dosage of brinase to produce autocytotoxicity was revealed.

Table 1

Occurrence of autocytotoxicity in relation to brinase therapy

Cases 1 and 2 were tested after an unexplained fall in white cell and platelet counts following whole blood transfusion.

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Av. dose mg</th>
<th>Previous remissions</th>
<th>Days to cytotoxicity</th>
<th>Duration (days) of cytotoxicity</th>
<th>Concomitant therapy</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. T. W.</td>
<td>M</td>
<td>22</td>
<td>ALL</td>
<td>18 100</td>
<td>0</td>
<td>8</td>
<td>12</td>
<td>Blood, VC</td>
<td>Peripheral remissionb</td>
</tr>
<tr>
<td>2. B. F.</td>
<td>M</td>
<td>7</td>
<td>ALL</td>
<td>7 50</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>Blood, VC</td>
<td>Complete remission maintained on VC and 6MP for 12 mo.</td>
</tr>
<tr>
<td>3. O. M.</td>
<td>F</td>
<td>3</td>
<td>ALL</td>
<td>16 20</td>
<td>0</td>
<td>0</td>
<td>28</td>
<td>Blood, 6MP, VCSteroids</td>
<td>Short complete remission</td>
</tr>
<tr>
<td>4. K. M.</td>
<td>F</td>
<td>45</td>
<td>AML</td>
<td>26 200</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>Warfarin</td>
<td>Short complete remission</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4 300</td>
<td>0</td>
<td>None</td>
<td>7</td>
<td>Warfarin</td>
<td>Clinical improvement</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 300</td>
<td>0</td>
<td>None</td>
<td>Warfarin</td>
<td>Clinical improvement</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 200</td>
<td>1</td>
<td>None</td>
<td>Warfarin</td>
<td>Clinical improvement</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 200</td>
<td>0</td>
<td>None</td>
<td>Warfarin</td>
<td>Clinical improvement</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 200</td>
<td>1</td>
<td>None</td>
<td>Warfarin</td>
<td>Clinical improvement</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25 250</td>
<td>0</td>
<td>None</td>
<td>Warfarin</td>
<td>Clinical improvement</td>
<td></td>
</tr>
<tr>
<td>5. P. M.</td>
<td>M</td>
<td>40</td>
<td>ALL</td>
<td>18 175</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>6MP, VC</td>
<td>Hematological remission</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14 150</td>
<td>0</td>
<td>3</td>
<td>6</td>
<td>Steroids</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28 150</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>L-Asparaginase</td>
<td>Complete remission maintained on VC, 6MP, and steroids</td>
</tr>
<tr>
<td>6. P. B.</td>
<td>M</td>
<td>6</td>
<td>ALL</td>
<td>22 30</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>None</td>
<td>Sepsis, hemorrhage, death.</td>
</tr>
</tbody>
</table>

a The abbreviations used in the table are: ALL, acute lymphoblastic leukemia; VC, vincristine; 6MP, 6-mercaptopurine; AML, acute myeloblastic leukemia.
b Case 1 died of hemorrhage following blood transfusion that produced thrombocytopenia and leukopenia 5 weeks after last positive in vitro autocytotoxicity test.
Chart 1. Patient P. M., male, 40 years old; acute lymphoblastic leukemia. 6-MP, 6-mercaptopurine; POLYS, polymorphonuclear leukocytes; LYMPHS, lymphocytes; BLASTS, lymphoblasts.

Case 5

P. M. (treated by Dr. M. I. Drury), was a 40-year-old man with acute lymphoblastic leukemia (Chart 1).

After brinase was given for 13 days, autocytotoxicity was produced and it lasted 12 days. When the in vitro test was negative for autocytotoxicity, blood transfusion had no effect on lymphocytes or platelets. When autocytotoxicity was 40% in vitro, however, blood transfusion lowered the lymphocyte count from 3,000 to 2,300 and lowered the platelets from 197,000 to 16,000. The lymphoblasts, 1,000 per cu mm (18% of total white cell count), all disappeared from the circulation. After a break of 7 days, a 2nd course of brinase, given for 13 days, produced autocytotoxicity which lasted 6 days.

The 3rd course of brinase produced autocytotoxicity after 11 days of therapy. It lasted only 3 days but the intensity was
marked, and 100% of the patient's lymphocytes were lysed completely. Brinase was continued for another 16 days as the patient went into remission and no more autocytotoxicity was found.

Comment. In combination with vincristine, prednisone, L-asparaginase, and 6-mercaptopurine, brinase was shown to produce autocytotoxicity on 3 separate occasions in the same patient.

Case 6

P. B. (treated by Dr. Conor Ward), was a 6-year-old boy with acute lymphoblastic leukemia. On February 12, 1971, following a transfusion of whole blood, this patient was started on brinase i.v., 2.5 mg/kg/day, and this treatment was continued through March 5. Autocytotoxicity was first noted on February 28 and lasted until March 8, varying from 40 to 60%. During brinase therapy, the platelet count rose from 20,000 to 70,000 and the neutrophil count rose from 400 to 2,000. A marrow aspiration on March 5 showed no change from the pretreatment aspirate. A transfusion of whole blood on March 5, when autocytotoxicity was 60%, produced a fall in platelet count from 65,000 to 20,000 and a fall in lymphocyte count from 2,000 to 1,000 within 1 hr. The platelet count recovered in 24 hr. There was a rise in neutrophil count; however, septicemia, which was first noted on March 1, combined with hemorrhage, complicated the situation and resulted in the patient's death on March 18.

Comment. This child, who had 4 previous remissions and was no longer responsive to conventional therapy, still produced autocytotoxicity.

Autocytotoxicity

Complement was required in all cases for lymphocytotoxicity and cytolysis. Rabbit complement was used in vitro because with human complement (AB serum) less cells were affected, especially when autocytotoxicity was low. Complement was fixed by the reaction at a temperature of 37°C. Estimations of complement levels in patients by the hemolysis screening test were within normal limits.

Cross-reactions with Homologous Lymphocytes

Autocytotoxic serum from Case 5 cross-reacted and killed more than 30% of the lymphocytes taken from Case 4, 2 cases of Hodgkin's disease, a case of multiple myeloma, and a case of lymphosarcoma. No cross-reaction occurred when nonautocytotoxic serum from Case 5 was used. Likewise, autocytotoxic serum did not cross-react with the patient's own lymphocytes taken outside the period of autocytotoxicity during remission or with the lymphocytes of his twin brother. Both had the same HL-A antigens. The lymphocytes of 20 nonmalignant cases and 5 cases in remission did not cross-react with the serum of Case 5.

DISCUSSION

In the 1st 3 cases previously reported, the presence of autocytotoxic antibodies was noted only by accident following brinase therapy. The detailed study of 3 more cases showed autocytotoxicity after 10 to 16 days of therapy. Because of the test system used, autolymphocytotoxic effects have been observed however, in the case of acute myeloblastic leukemia, blast cells were also found to be involved.

In 1954, Amos et al. (1) described lymphocytotoxic antibodies in the sera of allografted animals. In 1964, Terasaki and McClelland (16) devised their microdroplet method to screen for similar antibodies in the human. These lymphocytotoxins appeared in human sera after allogeneic immunization and were active at 37°C in vitro. In contrast, the lymphocytotoxins described by Terasaki et al. (17) in lupus, infectious mononucleosis, rubella, and measles are most active at 15°C in vitro and show no apparent activity in vivo. Thus, the autocytotoxicity found after brinase therapy, best observed at 37°C in vitro and in vivo following blood transfusion, appears to parallel closely that induced by allogeneic immunization.

The cross-reaction of autocytotoxic serum with lymphocytes of other patients not treated by brinase suggests that antibodies to brinase are not producing the lymphocytotoxicity, nor does brinase appear to act as a hapten. The lack of lymphocytotoxicity against the patient's own lymphocytes taken during remission suggests a difference between the lymphocytes in active disease and remission. This is further reinforced by the cross-reaction with lymphocytes from patients with myeloma, lymphosarcoma, and Hodgkin's disease. The lymphocytes of patients in remission did not cross-react. Much more investigation of a larger series of patients is needed before conclusions can be drawn.

Brinase interferes with cell membrane permeability in Landshutz tumor cells (14). Proteolytic enzymes in very low concentration have been shown by Burger (3) to change the cell surface and expose agglutination sites containing N-acetylglucosamine. Immunotherapy by proteolytic enzymes should make the leukemic cell more accessible to the antibody. Yoshida and Imai (23) have demonstrated autoantibodies to human leukemic cells in vitro after treatment of the cell preparations which probably exposed the antigenic sites.

We first used brinase in leukemia because of its ability to lyse cells in tissue culture and to lower antiplasmin levels. This allows the host's own plasmin (fibrinolysin) to act unopposed (19). Plasmin is a direct cytotoxic agent (22) and has been shown by Shimoyama et al. (13) to release lysosomal enzyme activity from tumor cells. The release of acid hydrolases that are localized in the lysosomes leads to autolysis of the cell (12) so that plasmin may be the main proteolytic enzyme used by the host to break down unwanted cells. Spitznagel and Allison (15) show that lysosomal labilizing agents act as adjuvants and enhance antibody formation. Thus brinase may play a double role by exposing antigens and enhancing antibody production.

The direct cytolytic action of Brinase occurs best during the 1st dose (18) and is not comparable to the complement-dependent cytotoxicity found in the serum of patients after completion of the course. According to the observations of Green et al. (6), cytotoxicity requires sufficient antibody and complement to be fixed by antigenic receptors at the cell wall. In 2 of our patients the autocytotoxicity test system required patients' serum and complement to be present during incubation of the cells at
37°, whereas in 4 others the patients' serum could be replaced by 0.9% NaCl solution, thus suggesting that the antibody was already cell bound. Complement, however, was fixed by the reaction.

The cells of the erythroid series do not appear to be involved; however, brinase in the absence of autocytoxicity increases the numbers of circulating platelets and polymorphonuclear leukocytes (8), possibly by the mechanism described by Burger (3) whereby proteolytic enzymes initiate cell division in normal cells.

The clinical effect of blood transfusion is puzzling because complement levels, when assayed by the hemolysin test, were normal in the patients. It is possible that the allogeneic blood supplies a toxic factor similar to that found in rabbit complement serum.

Brinase is a potent fibrinolytic agent with a direct action used to dissolve thrombi and to free cannulas in renal dialysis units (5). We have given single infusions of brinase to 137 patients and have observed no toxic effects on normal cells. Repeat courses can produce anaphylactic reactions that are controllable and do not interfere with the fibrinolytic activity of brinase (18).

Previous multiple blood transfusions do not seem to play a role in the production of the autocytoxic antibody. Its place in the HL-A system is still not worked out because HL-A typing of leukemic cells varies with disease state (9). The production of autocytoxicity, however, appears to be reproducible if adequate dosage of brinase is given for a sufficient time. Autocytoxicity can be produced during combined therapy with other drugs, as in Case 5 (Table 1), and after previous remissions, as in Cases 1 and 6. Thus, patients need not be deprived of other forms of antileukemic therapy while brinase is being investigated as an immunotherapeutic agent.

ACKNOWLEDGMENTS

Brinase was kindly donated by Astra AB, Sweden, through the courtesy of Dr. P. Frisch. Plasmin was prepared at Kabi AB, Sweden, by Dr. R. Lundén from retroplacental blood collected by the labor ward staff of the Irish Maternity Hospitals. The adult bone marrow smears were evaluated by Dr. J. D. Curran, Dr. R. J. W. Ryder, and Professor P. D. Holland.

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