The fast-acting and physiologically most important inhibitor of plasmin in human plasma is a recently discovered and purified $\alpha_2$-glycoprotein with a molecular weight of 65,000–70,000 daltons occurring at a concentration of 1 $\mu$M. The inhibitor rapidly forms a completely inactive 1:1 stoichiometric complex with plasmin through reaction with the B chain (light chain) of the enzyme, which contains the active center. It also reacts with trypsin and very slowly with urokinase and with some other enzymes in purified systems, but its role in vivo as an inhibitor of proteases other than plasmin seems negligible. Antiplasmin is the only plasma protein that can inhibit the fibrinolysis associated with transformed or malignant cells. The plasmin-antiplasmin complex contains neoantigenic structures not present in the parent molecules that may form the basis of immunochemical methods for detecting activation of the fibrinolytic system in blood.

Recently, a new and physiologically important inhibitor of plasmin was identified in human plasma by three different groups.1,4 This inhibitor has been called antiplasmin,1,2,4 $\alpha_2$ plasmin inhibitor,3 or primary plasmin inhibitor.4

Upon activation of plasminogen in plasma, the formed plasmin is first preferentially bound to antiplasmin. Only upon complete activation of plasminogen (concentration in plasma 1.5 $\mu$M), resulting in saturation of the antiplasmin (concentration in plasma 1 $\mu$M), is the excess plasmin neutralized by $\alpha_2$-macroglobulin.2 In the presence of normal concentrations of these two inhibitors, the other plasma protease inhibitors do not play a role in the inactivation of plasmin.2,6

During the last year, a number of studies have been performed on the characterization and function of this inhibitor. These studies will be briefly reviewed here.
Identification

Plasma and serum exert a very important inhibitory effect on plasmin. There are at least five well-defined plasma proteins that inhibit plasmin in a purified system, namely, \( \alpha_2 \)-macroglobulin, \( \alpha_1 \)-antitrypsin, inter-\( \alpha \)-trypsin inhibitor, antithrombin III-heparin complex, and \( C_1 \)-esterase inhibitor. However, the role of these inhibitors in the inactivation of plasmin formed in its natural environment, plasma, is not well understood. It has long been accepted that there are essentially two functionally important plasmin inhibitors in plasma, an immediate reacting one and a slow-reacting one,\(^7\) identical with \( \alpha_2 \)-macroglobulin and \( \alpha_1 \)-antitrypsin, respectively.\(^8,9\)

Müller\(^{6,10} \) studied the activation products of human plasminogen in post-mortem and urokinase-activated human plasma and found a compound that reacted with antiplasminogen antisera but not with antisera against the known protease inhibitors. One of the explanations he suggested was that this compound might represent plasmin in complex with a strong (unknown) inhibitor. Müller and Clemmensen\(^4 \) later purified this compound and provided unequivocal evidence for the existence of a plasmin inhibitor in plasma, different from the known protease inhibitors, that they called the primary plasmin inhibitor.

Aoki and von Kaula\(^{11} \) identified a fibrinolytic inhibitor in human serum that they thought acted mainly at the level of plasminogen activation. Later work by Moroi and Aoki\(^1 \) indicated, however, that this inhibitor acted primarily at the level of formed plasmin, and it was therefore called \( \alpha_2 \)-plasmin inhibitor.

During our studies on the turnover of labeled plasminogen during fibrinolytic therapy, we observed the formation in vivo of radioactive complexes that were initially assumed to represent plasmin-\( \alpha_2 \)-macroglobulin and plasmin-\( \alpha_1 \)-antitrypsin complexes.\(^12\) Further work, however, indicated that the presumed plasmin-\( \alpha_1 \)-antitrypsin complex did not react with antisera against \( \alpha_1 \)-antitrypsin. By isolating this complex and producing antibodies against its inhibitor part, we obtained positive evidence for the existence of a new fast-acting plasmin inhibitor in human plasma, which we called antiplasmin.\(^1,12\)

With the use of this antiserum it has since been shown that antiplasmin and the proteins purified by Moroi and Aoki\(^1 \) and by Müller and Clemmensen\(^4 \) are identical.

Gallimore\(^{13} \) partially purified a fibrinolytic inhibitor from human serum that he called inter-\( \alpha \)-antiplasmin. Recent data\(^{14} \) indicate that this preparation is a mixture of antiplasmin and the inhibitor of plasminogen activation described by Hedner,\(^{15} \) which in turn is different from antiplasmin.\(^{16} \)

Purification

To date four methods have been described for the purification of antiplasmin from plasma.

Moroi and Aoki used a combination of ammonium sulfate precipitation, DEAE-Sephadex chromatography, affinity chromatography on insolubilized plasminogen, and hydroxylapatite chromatography of plasminogen-depleted plasma and purified the inhibitor to homogeneity.\(^1 \) Wiman and Collen developed a modified purification method consisting of chromatography on in-
FAST-ACTING PLASMIN INHIBITOR

solubilized plasminogen followed by chromatography on DEAE-Sephadex and insolubilized concanavalin A, which resulted in a higher yield and a more stable inhibitor preparation. Müllertz and Clemmensen partially purified the inhibitor by ammonium sulfate precipitation, DEAE-Sephadex chromatography, chromatography on insolubilized concanavalin A, gel filtration, and further DEAE-Sephadex chromatography. Collected isolated the inhibitor (mostly in an inactive form, however) by immunoabsorption chromatography and gel filtration.

Quantitative Determination

The inhibitor can be accurately determined in plasma either with enzymatic assays based on the very fast inhibition of plasmin or with immunochemical assays using specific antisera. In the enzymatic assays, residual plasmin can be measured with natural or with synthetic substrates. The normal level of the inhibitor in plasma is around 70 mg/liter plasma, or 1 μM.1,3

Physicochemical Properties

Antiplasmin is a single-chain glycoprotein with a molecular weight of 65,000–70,000 daltons as estimated by sodium dodecyl sulfate (SDS)-gel electrophoresis and ultracentrifugation. On electrophoresis it migrates as an α2-globulin. The amino acid composition reported by Moroi and Aoki and that reported by Wiman and Collen correspond well for most amino acids, and the estimated carbohydrate content of the molecule was determined as 11.7% and 13.7%, respectively. The inhibitor is immunochemically different from α1-antitrypsin, α2-macroglobulin, C1-esterase inhibitor, antithrombin III, α1-antichymotrypsin, and inter-α-trypsin inhibitor and from the inhibitor of plasminogen activation described by Hedner and co-workers. The inhibitor is stable in solution above pH 6.3 but is rapidly inactivated below pH 6.0. The NH2-terminal amino acid sequence is Asn-Gln-Glu-Gly-, and the sedimentation constant is 3.4.3

Interaction With Enzymes

In purified systems and in plasma, antiplasmin forms a 1:1 stoichiometric complex with plasmin that is devoid of protease or esterase activity and that cannot be dissociated by denaturing agents such as urea, guanidine HCl, or dodecyl sulfate. SDS-gel patterns indicate that complex formation occurs by strong interaction between the light chain of plasmin and the inhibitor. Moroi and Aoki recently claimed that the complex between the inhibitor and the light (B) chain of plasmin is dissociated by hydrazine, indicating that there is an ester bond between the active-site seryl residue of plasmin and antiplasmin. A polypeptide with a molecular weight of 11,000–14,000 daltons may be released after formation of the complex. This finding has led to the assumption that inhibition occurs by hydrolysis of a specific peptide bond in the inhibitor followed by esterification between the newly formed COOH-terminal residue of the inhibitor and the active-site serine residue of plasmin. It was shown that the release of this polypeptide is not an essential step in complex formation but the result of hydrolysis of the complex by free plasmin.
Immunologic Abnormalities in an Animal Model of Chronic Hypoplastic Marrow Failure Induced by Busulfan

By C. A. J. Pugsley, I. J. Forbes, and A. A. Morley

The immunology of chronic hypoplastic marrow failure (CHMF, aplastic anemia) was studied in an experimental murine model of the disease induced by busulfan. B lymphocytes of peripheral blood, spleen, and bone marrow were reduced to 30%-40% and T lymphocytes of thymus, spleen, marrow, and blood were decreased to 20%-70% of control values. IgG and IgM antibody titers to sheep red blood cells were reduced to one-third of control levels, and splenic IgG, but not IgM, plaque-forming cells were fewer on day 7 after antigen stimulation. The proliferative responses to phytohemagglutinin or concanavalin A were reduced in cultures of peripheral blood lymphocytes, splenic lymphocytes, and thymocytes, and cutaneous delayed-type hypersensitivity induced by dinitrofluorobenzene was not detected in mice with CHMF. The results demonstrate disturbance of a variety of cellular and humoral functions and suggest that the disturbance was due to quantitative and possibly qualitative abnormalities of the cell types subserving these functions. The results suggest that residual cell injury, the lesion underlying experimental CHMF, is not confined to the myeloid stem cell but also involved cells of the lymphoid series.

Chronic Hypoplastic Marrow Failure (CHMF, aplastic anemia) is commonly conceived of as a syndrome characterized by failure of delivery of erythrocytes, granulocytes, and platelets by a marrow that is hypoplastic but that shows no evidence of any other disease. This concept suggests that the primary disorder is restricted to the myeloid stem cell line, but there is some evidence that this is only partially true. Lymphocytopenia has been recognized for some time, deficiencies of B lymphocytes and monocytes have recently been reported, and disturbances of humoral and cellular immune function have been described.

An experimental model of CHMF can be produced in mice by treating them with a short course of busulfan. The treatment causes a type of damage to the stem cells of the marrow that has been termed "residual injury." The characteristics of residual injury are that it is permanent, there is impairment of stem cell proliferation leading to moderate marrow failure, and complete failure of stem cell proliferation may ensue, leading to severe marrow failure and death. Studies to date indicate that this experimental model closely resembles the human disease.

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The present study into the immune system in experimental CHMF was undertaken for a number of reasons: first, to test further the validity of experimental CHMF as a model for the human disease; second, to analyze the immune disturbances in more detail than would be possible in man; and third, to provide information on whether or not the lymphomyeloid stem cell, as opposed to the myeloid stem cell, was involved in the disease. A final reason was to determine if the residual damage could affect cells other than those of the marrow, since this would have implications regarding the possible widespread biologic importance of the phenomenon.

MATERIALS AND METHODS

Animals

CHMF was produced by administration of four doses of busulfan to virgin female Balb/c mice as previously described.7 Injections of 20, 20, 10, and 10 mg/kg were given intraperitoneally at intervals of 2 wk, and the mice were then allowed to recover for at least 2 mo after the last dose before being studied. At the time of study the mice appeared well, with no obvious signs of infection, although from previous studies9 it was known that their marrows still manifested "latent" or "residual" injury.

Preparation of Tissues

Spleens and thymuses were teased apart using fine needles and the cells suspended in phosphate-buffered saline (PBS) supplemented with 5%, fetal calf serum. Bone marrow cells were expelled from tibias and suspended in PBS. Peripheral blood lymphocytes were obtained by layering heparinized blood onto Ficoll-Hypaque gradients.

Measurement of Cell Numbers

Nucleated cells. These were counted with a hemocytometer.

B lymphocytes. B lymphocytes were enumerated by measuring the proportion of viable cells bearing surface immunoglobulin. A modification of the method of Taylor et al.2 was used. Cell suspensions were treated with Tris-buffered NH4Cl to lyse red blood cells.12 Dead cells were removed by suspending the cells in glucose:PBS (nine parts 5.5% glucose to one part PBS) and filtering the suspension through a small plug of cotton wool. Lymphocytes were incubated at 4°C with rabbit anti mouse gamma globulin conjugated with fluorescein isothiocyanate (ICN Pharmaceutical) and were then washed four times before examination under ultraviolet light using a Leitz Ortholux II microscope. Between ISO and 500 cells per slide were examined, and the absolute number of B lymphocytes was calculated from the percentage as determined by immunofluorescence and the total cell count.

T lymphocytes. Thymus-derived lymphocytes were estimated by the use of anti-Thy-I antisera in the "two-stage" cytotoxicity test using trypan blue.13 Guinea pig serum was used as the source of complement after adsorption with agar to reduce cytotoxicity.14 Background cytotoxicity was 5%-10%, for all cell populations examined. Tests were performed in triplicate on mouse thymus, spleen, bone marrow, and peripheral blood.

Immunization With Sheep Red Blood Cells (SRBC)

Mice were injected intraperitoneally with 4 x 10 SRBC on day 0 and groups of 4 or 5 mice were killed on days 3, 5, 7, 10, 14, and 21. Mice were bled by cardiac puncture, the serum was separated for antibody determination, and the spleens were assayed for absolute number of IgM and IgG plaque-forming cells (PFC). The number of splenic IgM PFC was determined by plating quadruplicate aliquots of spleen cells in the presence of SRBC.15 The number of IgG PFC was measured in quadruplicate using the method of Nordin et al.16 Briefly, plates were treated with concanavalin A (con A) (Calbiochem, grade A) and the IgG plaques developed by rabbit anti mouse IgG serum (ICN Pharmaceuticals) at a 1:100 dilution.

molecular events occurring during plasminogen activation by malignant proteases in purified systems.

Plasmin-Antiplasmin Complex: Its Determination and Value as an Indicator of Activation In Vivo of the Fibrinolytic System

Activation of the fibrinolytic system results in formation of plasmin, which has a short lifespan in the blood, since it is very rapidly bound to and neutralized by antiplasmin. If plasmin-antiplasmin complex formation were associated with the emergence of structural or conformational alterations that render this complex antigenically distinct from the precursor molecules, immunochemical quantitation of such neoantigens could constitute a means of measuring activation of the fibrinolytic system in vivo.

The plasmin-antiplasmin complex was isolated from plasma in which the fibrinolytic system was activated by affinity chromatography and gel filtration.2 This purified material was used for the production of antisera in rabbits by conventional immunization procedures. The occurrence of precipitating antibodies to structures present in the complex but not in the precursor molecules was demonstrated by immunodiffusion.30 With the use of a passive hemagglutination inhibition immunoassay using tanned red cells coated with purified plasmin-antiplasmin complex it was shown that it was possible to measure the plasmin-antiplasmin complex directly in human plasma on the basis of immunochemical quantitation of its neoantigens.31

For the study of the occurrence in vivo and clinical relevance of this complex, we developed a simple latex agglutination test for its rapid quantitation in plasma.32 In this method, polystyrene (latex) particles are coated with purified gamma globulins from the absorbed sera.

The purified complex was found to cause a clear agglutination of the particles at a concentration of 0.1-0.2 mg/liter; purified plasminogen and antiplasmin were more than 100 times less reactive. Activation of fresh human plasma with urokinase caused progressive generation of agglutinating activity up to a plasma dilution of 1:480. Intravenous injection of streptokinase into patients resulted in an increase of the plasmin-antiplasmin titer to at least 1/240. The titer remained high during the first 3 hr after the injection and was still elevated after 24 hr, indicating that the half-life of this complex in plasma is several hours.

Of 101 male and 23 female control subjects, only 3 males had a plasmin-antiplasmin titer above 1/16. Of 230 hospitalized patients, 25 had plasmin-antiplasmin titers of 1/40 or more. Most of these patients had diseases frequently associated with coagulation or fibrinolysis in vivo, but only one of them showed diffuse intravascular coagulation detectable by routine methods. In those patients without increased plasmin-antiplasmin titers, none of the hemostasis analyses was indicative of coagulation or fibrinolysis in vivo. Of eight patients with diffuse intravascular coagulation due to various causes, seven had plasmin-antiplasmin titers of 1/80 or 1/160.

From these findings it was concluded that activation of the fibrinolytic system in vivo, either directly or secondarily to coagulation in vivo, is associated with the appearance of circulating plasmin-antiplasmin complexes that can be directly assayed in plasma on the basis of their neoantigenic expression.
The IgM response to SRBC was determined by measuring the titer of hemolytic antibody.\(^{17}\) The antibody titer was regarded as that dilution giving 100% lysis. The IgG response to SRBC was measured by incubating 0.1 ml serum with an equal volume of SRBC for 1 hr at 37°C. The red cells were then washed four times in saline and finally resuspended in a suitable dilution of rabbit anti-mouse IgG. The titers were expressed as the last dilution of serum giving total hemagglutination. As an additional control, it IgM antibody was inactivated by pretreatment with 0.1 M 2-mercaptoethanol, but this had no effect on any day except day 3, when it led to a slight decrease in titer. Plaque size was determined by microscopic (x 10) determination of the diameter of plaques formed from single cells on day 5.

### Lymphocyte Transformation

Splenocytes or thymocytes were obtained for each experiment by removing and pooling spleens or thymuses from 3-5 mice. Whole blood lymphocyte cultures were derived from groups of 4 mice, whereas purified lymphocyte cultures were derived from groups of 8-10 control mice and 11-16 mice with CHMF. Thymus cells were cultured with concanavalin A, spleen cells and peripheral blood lymphocytes were cultured with phytohemagglutinin (PHA) (Wellcome Pharmaceuticals, HA 15 for splenocytes and HA 17 for peripheral blood lymphocytes).

Cells from thymus, spleen, and peripheral blood were cultured in RPMI-1640 medium (Commonwealth Serum Laboratories) containing bicarbonate buffer and supplemented with 6% heat-inactivated human serum, 2 mM glutamine, and L-arginine 150 μg/ml.\(^9\) Cultured splenocytes (3 x 10⁶ cells/tube) were harvested by filtration onto Whatman glass fiber paper. Each filter was washed three times with ice-cold saline, 5% trichloroacetate (TCA), water, and methanol in that order. Thymus and purified blood lymphocytes were cultured in microtiter trays (Linbro tissue culture grade). Quadruplicate 0.2-mi cultures were seeded with 4 x 10⁶ cells that were ultimately harvested using a multiple automatic sample harvester. All cell cultures were grown for 72 hr at 37°C in an atmosphere of 5% CO₂ and pulse labeled for the last 4 hr with 1 sCi ³H-thymidine (specific activity 5 Ci/mmole: Radiochemical Centre, Amersham).

Whole blood cultures were performed by the method of Han and Pauly.\(^{21}\) Quadruplicate cultures were established in RPMI-1640 supplemented with 10% bovine serum albumin (Cohn Fraction V, Sigma Chemicals Lot SSC-0074), glutamine, arginine, and antibiotics as above. Cultures containing 0.075 ml of whole blood were harvested on the third day, when the peak mitogenic response had been obtained.

### Delayed-type Hypersensitivity (DH)

This phenomenon was measured in two experiments using the radioisotopic method of Vadas et al.\(^{21}\) The DH response was measured isotopically in the ears, and the severity of the response was assessed by the magnitude of the ratio of the radioactivity between the left and right ears. For each experiment, normal mice and mice with CHMF were divided into one test and two control groups, each containing 4-6 mice. The antigen used was dinitrofluorobenzene (DNFB) in a carrier solution of acetone:olive oil (1:1). The test group was challenged and sensitized with DNFB. The first control group was sensitized with DNFB but challenged with carrier solution, and the second control group was sensitized with carrier solution but challenged with DNFB. The method of sensitization and challenge and the time of injection of 125Iodo²deoxyuridine (specific activity 90-110 mCi/mg: Radiochemical Centre, Amersham) and of ear removal were as described by Vadas et al.\(^{21}\)

### Significance Testing

The statistical techniques used were the Wilcoxon rank sum and x² tests and analysis of variance.\(^{22}\)

### RESULTS

#### Cell Numbers

In Table I are shown the number of total nucleated cells in spleen, thymus, and marrow; Fig. 1 shows the number of B lymphocytes in spleen, marrow, and

### REFERENCES


27. Danis K, Reich E: Inhibitors of plasminogen activation, in Reich E, Rifkin DB, Shaw E (eds): Proteases and Biological Control.
Table 1. Number of Nucleated Cells/Organ

<table>
<thead>
<tr>
<th>Organ</th>
<th>CHMF Control</th>
<th>CHMF Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>1.30 (1.39-1.21)</td>
<td>2.03 (2.11-1.94)</td>
</tr>
<tr>
<td>Thymus</td>
<td>1.03 (1.32-0.80)</td>
<td>4.18 (5.07-3.45)</td>
</tr>
<tr>
<td>Marrow</td>
<td>2.7 (3.3-2.3)</td>
<td>9.8 (10.3-9.3)</td>
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The results are based on 20 mice in each group and are expressed as the logarithmic mean with the mean plus 1 SE and the mean minus 1 SE in parentheses. For each organ there were significantly (p < 0.01) fewer cells in mice with CHMF.

Blood and T lymphocytes in thymus, spleen, marrow, and blood. The number of B lymphocytes in thymus was too low to score with accuracy. In Fig. 1 each point for thymus, spleen, and marrow refers to the results from one mouse, whereas each point for B lymphocytes in blood is derived from blood pooled from two or three mice and each point for T lymphocytes in blood is derived from blood pooled from six to eight mice. The logarithmic mean number of B lymphocytes in mice with CHMF was 30-40% of control values, and the logarithmic mean number of T lymphocytes was 20-70%.

All of the low results for mice with CHMF were statistically significantly less than results for control mice, except for the number of T lymphocytes in peripheral blood. In this instance, although the observed logarithmic mean in mice with CHMF was 58% of the mean observed for control mice, the difference did not quite reach statistical significance (0.1 > p > 0.05, two-tailed Wilcoxon test).

Response to Antigenic Stimulation by SRBC

The IgM and IgG responses to SRBC were measured in three separate experiments. The pooled results are shown in Fig. 2. The numbers of IgM and IgG plaque-forming cells (PFC) were measured in two separate experiments;
Fast-acting plasmin inhibitor in human plasma

D Collen and B Wiman