Pathologic Effects of Plasma From Patients With Thrombotic Thrombocytopenic Purpura on Platelets and Cultured Vascular Endothelial Cells

By Edward R. Burns and Dorothea Zucker-Franklin

The pathologic hallmarks of thrombotic thrombocytopenic purpura (TTP) include endothelial cell proliferation and subendothelial hyalin deposits in the microvasculature leading to symptomatic thrombotic occlusions. Plasma or sera from three consecutive patients with TTP were subjected to multiple analyses to determine whether they induce endothelial injury and/or platelet activation, two pathogenic mechanisms that may account for this disorder. Sera were utilized in a microcytotoxicity assay against cultured human umbilical vein endothelial cells (EC). These cells were assessed ultrastructurally and with immunofluorescence techniques to ascertain the nature of inflicted cell damage. Control plasmas were obtained from healthy volunteers as well as patients with immune complex disease. The adult hemolytic uremic syndrome. In the presence of TTP serum, cell kill of 3H-proline-labeled EC averaged 42% versus 8.6% for control sera. Cytotoxicity induced by an IgG fraction of TTP sera averaged 70% versus 16.8% for control IgG. Removal of IgG by immune precipitation diminished cytotoxicity by 70%. Using indirect immunofluorescence, IgG was detected on EC incubated with TTP serum but not on EC treated with control serum. Ultrastructural changes became apparent within 30 min after exposure of cultured EC to TTP serum. Virtually every cell developed numerous cytoplasmic inclusions rarely seen in EC in the presence of normal serum. Prolonged incubation with the TTP serum led to progressive cytolysis, terminating with complete cytoplasmic and nuclear degeneration.

Plasma from all three patients with TTP caused spontaneous aggregation of normal washed platelets as monitored by aggregometry. No spontaneous aggregation occurred in response to control plasmas. These results indicate that the sera of the three TTP patients studied were able to mediate time-dependent immune destruction of human cultured endothelial cells and that their plasmas were capable of causing spontaneous aggregation of normal human platelets in vitro. It would seem likely that these mechanisms are also operative in vivo to produce the endothelial destruction as well as the thrombotic vascular occlusions seen in this disorder.

Although the clinical manifestations of thrombotic thrombocytopenic purpura (TTP) have been well categorized, the pathogenesis and treatment of this disorder are less well defined. The thrombocytopenic and microangiopathic hemolytic anemia that are associated with fever and abnormalities of renal and neurologic function are readily explained by the pathology of the syndrome. Most notable are thrombotic occlusions of the arterioles and capillaries of the brain, kidneys, and systemic microcirculation. On the other hand, the underlying mechanisms accounting for diffuse thrombosis in the absence of intravascular coagulation remain elusive.

Recently, attention has been focused on the in vitro effects of plasma from patients with TTP on cultured human endothelial cells as well as on human blood platelets. These studies were prompted by observations of biopsy material from affected patients that have shown endothelial proliferation (see Gore) and subendothelial hyalin deposits. Both fibrin and IgG have been identified as constituents of the subendothelial deposits, which may represent the primary lesion or may be the result of focal platelet deposition. Because of the controversy surrounding the very existence of a toxic plasma factor in this syndrome, we studied the effects of plasma from three patients with TTP on platelets and cultured vascular endothelial cells. The results are consistent with the hypothesis that immunologic destruction of endothelial cells and activation of normal platelets by TTP plasma play primary roles in the pathogenesis of this disorder.

Materials and Methods

Plasma Sources

Plasma was obtained from three patients presenting with the classic clinical pentad of TTP. Each had marked thrombocytopenia and anemia with normal coagulation parameters. Their blood smears showed schistocytes, and bone marrow aspirates demonstrated erythroid and megakaryocyte hyperplasia. Primary therapy consisted of plasmapheresis with concomitant fresh-frozen plasma infusion. Antiplatelet drugs were added as necessary for remission maintenance. None of the patients had received any blood products before collection of their acute phase plasma during first-pass plasmapheresis.
Patients

Patient 1 (CC) was a 60-yr-old woman who achieved remission 3 days following initiation of therapy. Her clinical status fluctuated in relation to her plasma infusions, until discharge after 3 wk. Biweekly outpatient plasmaphereses were necessary to maintain a normal platelet count. She died unexpectedly after 6 mo while having a routine plasmapheresis. An autopsy was unrevealing.

Patient 2 (HD) was a 45-yr-old man who achieved remission 5 days after beginning therapy and has remained symptom-free for over 2 yr without further therapy.

Patient 3 (SG) was a 78-yr-old woman who experienced a fulminating course complicated by acute renal failure and coma. She died after 4 days of therapy without achieving remission.

Controls

These consisted of 10 normal laboratory volunteers, two thrombocytenic patients with chronic renal failure and circulating immune complexes, and one patient with adult hemolytic uremic syndrome.

Immunologic Profiles

Serum protein electrophoresis and immunoelectrophoresis patterns were normal in all patients and controls. There were no cryoglobulins present in any samples tested. The presence of soluble immune complexes were investigated by treating serum with polyethylene glycol (PEG) to effect their precipitation. This induces a turbidity that can be measured spectrophotometrically at OD 450. Only one patient (patient 1) and none of the controls had an OD level that was even in the upper range of normal for our patient population (normal 0–0.3). This value of 0.3 was considered insignificant because electrophoresis of the patient’s redissolved PEG precipitate revealed only a negligible increase in polyclonal IgG, indicating no specific immunologic abnormality. All other samples had values of zero.

Plasma Collection

Plasmas were collected into blood collection bags containing citrate, phosphate, dextrose (Fenwall, Morton Grove, Ill.) during therapeutic or voluntary plasmapheresis. Following fractionation into 10-ml aliquots, they were either heat-inactivated at 56°C for 30 min or not inactivated, frozen, and stored at −20°C. Some samples were recalified and the formed fibrin clot processed in a tissue homogenizer then centrifuged for 30 min at 30,000 rpm in a Sorvall RC-2 centrifuge kept at 4°C. The supernatant serum was aspirated and an aliquot reacted with bovine thrombin (Parke Davis, Detroit, Mich.) to assess whether any clottable fibrinogen remained. No clot formed in any instance. The use of serum in the microcytotoxicity tests and electron microscopy studies was mandated by preliminary observations that patient plasma exposed to cultured endothelial cells clotted, thereby interfering with the assays. Processed serum was frozen and stored as above. For some controls, whole blood was collected into glass tubes, allowed to clot at 37°C, and centrifuged prior to collection of serum.

Preparation of IgG Fractions

IgG fractions of control and patient sera were prepared by passage of dialyzed sera through a diethylaminoethyl cellulose column (Whatman DE-52) equilibrated with 0.01 M phosphate buffer, pH 7.4. The IgG fraction was then lyophilized and redissolved in phosphate-buffered saline to achieve standard concentrations of 1 mg/ml.

Cytotoxicity Assay

A microcytotoxicity test of TTP serum against human endothelial cells was employed. Human umbilical vein endothelial cells were grown as described using Medium 199 with 20% newborn calf serum (NCS) as growth medium. These cells met morphological criteria for endothelial cells having Weibel-Palade bodies. Second passage cells were detached from culture dishes with a 0.2% collagenase ( Worthington, Freehold, N.J.) solution and replaced in Minimal Essential Medium–20% NCS without proline in the presence of 3H-proline. After incubating overnight at 37°C in a CO2 incubator, the cells were again detached and replated onto Microtest II culture dishes (Falcon, Cockeysville, Md.), at a density of 4000 cells/well in 0.1 ml Medium 199–20% NCS. All samples were run in triplicate. After the cells attached to wells precoated with a 0.2% gelatin solution, 20% of serum or IgG fractions were added and the plates incubated an additional 16 hr, a time derived from preliminary studies that evidenced maximal cytotoxicity. After extensive washing to remove loose cells, the residual cells were solubilized with hydroxide of hyamine (Packard, Downer’s Grove, Ill.) and their radioactivity quantified using a Beckman liquid scintillation counter. This technique thus measured actual cell kill as opposed to only release of isolate. Cytotoxicity was expressed according to the following formula:

\[
\text{Cytotoxicity} = \frac{\text{Untreated cells cpm} - \text{Patient (or control) cpm}}{\text{Untreated cells cpm}} \times 100
\]

Mean cytotoxities ± SEM were calculated for patients and controls. Data from all three patients were grouped together and compared to controls. The t test was used with the null hypothesis comparing average patient cytotoxicity with average control cytotoxicity. Collagenase treatment of the endothelial cells caused no significant spontaneous cytotoxicity over control nondetached cells.

Immunofluorescence

Endothelial cells were grown on glass coverslips and washed repeatedly with serum-free medium before use. They were incubated for 30 min with 100% control or patient sera or IgG fractions, washed extensively with serum-free medium, and fixed with absolute ethanol. This was followed by incubation with monospecific fluorescein-conjugated goat anti-human IgG (Meloy, Elk Grove Village, Ill.) for 30 min at room temperature and thorough washing with phosphate-buffered saline. The coverslips were mounted upside down onto glass microscope slides with Elvanol mounting medium (Dupont, Wilmington, Del.). Slides were viewed with a Leitz Ortholux II fluorescent microscope and graded for fluorescence intensity using a scale of 0 to +++; grading was done by an independent observer on coded specimens to prevent bias.

Electron Microscopy

Cells grown in 60 sq mm polystyrene dishes (Corning, Corning, N.Y.) were exposed to an environment of 50% medium–50% serum (either test or control) for varying times ranging from 30 min to 24 hr. Following a double wash with fresh serum-free medium, they were detached from the dishes and fixed in suspension with 3% phosphate-buffered glutaraldehyde. They were postfixed with 2% osmium tetroxide and stained en bloc with 0.5% uranyl acetate, dehydrated with ethanol, and embedded in Epon 812. Ultrathin sections were obtained with an LKB I microtome and examined with a Siemens Elmiskop I electron microscope. Sections representing normal endothelial cells or cells incubated with control or patient plasmas were examined “blindly” for ultrastructural changes.

Platelet Aggregation Studies

Washed normal platelets incubated with heterologous control or patient plasma were studied using a modification of the method of Lian et al. Briefly, 0.3 ml of patient or control plasma were warmed...
to 37°C in a Chronolog aggregometer cuvette before the addition of 750 x 10^3 platelets/ml in 0.2 ml of calcium-free Tyrode’s buffer, pH 7.3. Spontaneous aggregation, without the addition of standard aggregation agents, was then monitored. To insure platelet viability, aliquots of platelets were assayed for their ability to aggregate in response to adenosine diphosphate using standard methodologies. In each experiment, following addition of plasma, aliquots of platelets were examined with a phase microscope to verify whether or not aggregation had occurred.

Red Cell Agglutination Studies

Control and TTP sera were reacted with a panel of 4 lots of type O human erythrocytes, each with a distinctly defined array of surface antigens. Two drops each of serum and a 6% red cell suspension were incubated for 15 min at room temperature and for 20 min at 37°C in the presence of 2 drops of 22% bovine serum albumin. Agglutination was looked for microscopically. Following this, 2 drops of rabbit anti-human globulin (Coombs reagent) were added and the mixture examined with a phase microscope to verify whether or not agglutination, both macroscopically and microscopically. The supernatant was also examined for evidence of hemolysis.

RESULTS

Cytotoxicity Data

Sera from all three patients were cytotoxic for cultured endothelial cells. As seen in Table 1, in 6 experiments, each using a different line of endothelial cells, TTP patients averaged a 42% cytotoxicity index versus 8.6% for controls. In each of the 6 cell lines tested, average patient cytotoxicity was greater than that of controls (range: 2–16 times greater). This was significant at the 0.05 level in 3 of the cell lines and not significant \((p > 0.09)\) in the other 3. In 3 experiments in which cytotoxicity of untreated cells was measured at 1 hr, 3 hr, and 24 hr after cell attachment to the microtiter plate, spontaneous cytotoxicity measured less than 3%. There was no difference in cytotoxicity among the controls whether the serum was derived from normal whole blood, normal plasmapheresis plasma, renal failure, or hemolytic uremic plasmapheresis plasma. Cytotoxicity was abolished by heat inactivation of serum at 56°C for 30 min.

The factor(s) responsible for cytotoxicity could not be removed by equilibrium dialysis against 0.01 \(M\) phosphate buffer. Surprisingly, dialysis seemed to increase cytotoxicity of normal and patient sera despite reconstitution to starting volumes (data not shown). These findings were explored further by way of mixing studies in which equal volumes of dialyzed and undialyzed sera were preincubated for 1 hr before incorporation into the cytotoxicity assay. Neutralization of cytotoxicity occurred with one control and one of two patient samples tested. Similarly, normal serum neutralized cytotoxicity in only 1 of the 3 TTP patients studied. No explanation for these observations is available at the present time.

The IgG fractions isolated from the TTP patients were markedly cytotoxic (Table 1), whereas those from 10 normal controls were not. To verify that cytotoxicity of the IgG fraction was actually due to IgG and not an artifact of preparation, the patient Ig fractions were treated with equiweight aliquots of purified rabbit anti-human IgG antiserum. The resulting precipitate was removed by centrifugation and the supernatant tested in the assay system. The reduction in cytotoxicity achieved by depletion of IgG is shown in Table 2. The remaining cytotoxicity approximated that in controls.

Immunofluorescence

To further verify that the IgG fraction of the patients’ sera was involved in mediating cytotoxicity, purified control and patient IgG were reacted with endothelial cell monolayers and observed using an indirect immunofluorescence technique. As shown in Table 3, cells treated with buffer, control serum, or control IgG showed no fluorescence. Sera of two of the three patients caused strong immunofluorescence, and the purified IgG of all three patients demonstrated similar positivity (Fig. 1). Depletion of IgG by immune precipitation abolished fluorescence. Therefore, it may be concluded that the IgG derived from the serum of all three patients reacted specifically with the cultured endothelial cells.

Platelet Aggregation

The results of aggregation studies on two of these three patients have been reported elsewhere.\(^{12}\) These

### Table 1. Cytotoxicity Against Human Endothelial Cells

<table>
<thead>
<tr>
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<th>Serum</th>
<th>IgG</th>
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<tbody>
<tr>
<td>Patient 1</td>
<td>31.6 ± 9.5 (n = 6)</td>
<td>66.0 ± 6.0 (n = 5)</td>
</tr>
<tr>
<td>Patient 2</td>
<td>35.3 ± 7.2 (n = 6)</td>
<td>56.7 ± 23.7 (n = 3)</td>
</tr>
<tr>
<td>Patient 3</td>
<td>59.0 ± 14.6 (n = 6)</td>
<td>87.6 ± 1.5 (n = 2)</td>
</tr>
<tr>
<td>Mean</td>
<td>41.9 ± 8.6</td>
<td>70.1 ± 9.2</td>
</tr>
<tr>
<td>Controls</td>
<td>8.2 ± 3.5 (n = 8)</td>
<td>16.6 ± 0.7 (n = 10)</td>
</tr>
</tbody>
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Values represent mean percent ± SEM.

### Table 2. Effect of IgG Depletion on Percent Cytotoxicity

<table>
<thead>
<tr>
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<th>IgG Fraction</th>
<th>IgG Depleted</th>
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<tbody>
<tr>
<td>Patient 1</td>
<td>64.9</td>
<td>20.2</td>
</tr>
<tr>
<td>Patient 2</td>
<td>19.6</td>
<td>4.3</td>
</tr>
<tr>
<td>Patient 3</td>
<td>89.6</td>
<td>28.7</td>
</tr>
<tr>
<td>Mean</td>
<td>58 ± 20.5*</td>
<td>17.7 ± 7.2†</td>
</tr>
</tbody>
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*Mean ± SEM.
† \(p < 0.05\) when compared to IgG fraction.

### Table 3. Immunofluorescence of Treated Endothelial Cells

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Patient 1</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Patient 2</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Patient 3</td>
<td>+</td>
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were repeated with studies on the third patient as described in the methods section and illustrated in Fig. 2. Plasmas from all three patients caused spontaneous aggregation of normal donor platelets, confirming the results of the previously cited report. Plasmas from six normal volunteers did not possess any platelet aggregating activity. Phase microscopy verified aggregation of platelets as recorded by the aggregometer and did not reveal evidence of platelet lysis.

Red Cell Agglutination

Neither control nor any of the TTP sera caused red cell agglutination or hemolysis. The presence of the anti-human globulin did not facilitate any reaction.

Endothelial Cell Ultrastructure

Normal cultured endothelial cells fixed in suspension had an average diameter of 18 μ. As noted previously, these cells frequently show cytoplasmic vacuoles, which probably are due to imperfections of the in vitro culture technique. When vascular endothelial cells are grown in the presence of fibroblast growth factor, such vacuoles are not seen. In cells treated with normal serum, cytoplasmic and nuclear ultrastructure were intact regardless of the length of incubation or the source of control serum. Most cell sections did, however, possess 3–5 vacuoles (Fig. 3), measuring approximately 2 μ in diameter. Some were free of vacuoles and none showed extensive cytoplasmic vacuolization (Fig. 4).

In marked contrast was the morphology of the cells treated with TTP serum from all three patients. The earliest changes were seen after only 30-min exposure. These were striking enough to be readily noted by an uninformed observer and consisted of multiple 1–2 μ globular cytoplasmic inclusions. Almost all cells contained such inclusions, which numbered from 3 to 20 in each cell (Figs. 5 and 6). They were not membrane bound. Cells treated with control serum were virtually free of these inclusions. When present, they were extremely sparse (i.e., 1 or 2).

Incubation of endothelial cells with TTP serum beyond 30 min resulted in progressive cytoplasmic and nuclear degeneration. Vacuoles became numerous, i.e., 10–20/cell section. The cytoplasmic fibrillar material and endoplasmic reticulum seen in cells treated with control serum became unrecognizable, and the nuclear chromatin appeared degenerated. By 24 hr, most cells had become completely necrotic and were no longer distinguishable as endothelial cells (Fig. 7).

DISCUSSION

The pathology of thrombotic thrombocytopenic purpura is characterized by vessel occlusion with hyalin
Fig. 3. Survey electron micrograph of cultured human umbilical vein endothelial cells incubated for 30 min with serum derived from normal human subjects. Note that there is a moderate degree of vacuolization, a common finding in endothelial cell cultures under standard conditions. N, nucleus (×3600).

Fig. 4. Higher power view of a single endothelial cell taken from a specimen that had been incubated with normal serum. Note that the cytoplasmic area replete with the fine fibrils (arrow) often seen in these cells appears to be well preserved (×2500).
Fig. 5. Survey electron micrograph (x2600) of endothelial cells that had been incubated with TTP serum for 30 min. There are innumerable grey inclusions in every cell, only some of which are indicated by arrows. The area delineated by the rectangle is seen at higher magnification in Fig. 6. Fig. 6. High power view (x12,600) of a detail of the cell seen in the upper right corner of Fig. 5 shows globular inclusions to better advantage (arrows). A portion of the cerebriform nucleus is seen with 2 prominent nucleoli (N). Fig. 7. Endothelial cell incubated with TTP serum for 6 hr. The cytoplasm is completely necrotic, and the nucleus (N) appears devoid of chromatin (x4200).
deposits and associated focal endothelial proliferation. Any theory promoted to account for the pathogenesis of these findings must address the question of whether the initial event leading to vessel plugging is vascular damage with secondary platelet adhesion and aggregation or primary platelet aggregation with concomitant intraluminal and subendothelial fibrin deposition.

There is evidence to support both these theories. Lian and coworkers have reported that TTP plasma induces spontaneous platelet aggregation and that mixing TTP plasma with normal plasma reverses this in vitro effect. Furthermore, infusion of normal plasma into patients suffering from TTP has occasionally been successful in reversing the disease process. These observations lend credence to the theory that activated platelets play a primary role in the disease’s pathogenesis. On the other hand, two preliminary reports suggest that TTP plasma is cytotoxic to endothelial cells in vitro, thereby implicating primary endothelial injury as the important mechanism. To further complicate matters, both these theories have been questioned by one report showing no cytotoxic or aggregating activity for TTP plasma and another demonstrating platelet-aggregating activity in only one of two patients studied.

In the present study, the sera of three consecutive patients with TTP were shown to contain an IgG antibody apparently directed against endothelial cells. By immunofluorescence this antibody was observed to bind specifically to cultured human endothelial cells, and its destructive effect was demonstrated morphologically and by a standard isotope technique. Although cytotoxicity due to the presence of thrombin in the patients’ sera has not been ruled out, the correlation of specific IgG binding to endothelial cells by patients’ sera makes immune destruction a more plausible explanation. In addition, plasma from these patients caused aggregation of normal human platelets and effected these phenomena in the absence of circulating immune complexes. That the platelet aggregation induced by TTP plasma can also be mediated by immune mechanisms is suggested by the recent finding of elevated platelet-bound IgG in a patient with this disease.

The fact that erythrocytes treated with TTP serum did not agglutinate even in the presence of a polyvalent anti-human globulin is strong in vitro evidence that there was no binding of the TTP IgG to erythrocytes. This suggests some specificity of the antibody to endothelial cells and, possibly, platelets.

Recently, complement-dependent endothelial cell damage has been demonstrated in mice in a disease similar to TTP. Furthermore, human antibodies directed against certain DRw antigens have been shown to cross-react with cultured endothelial cells and cause complement-dependent cytotoxicity. Since the DRw antigens are present on human endothelial cells, it is possible that TTP may represent an HLA-related autoimmune phenomenon.

The finding that dialysis of both normal and TTP sera caused an increase in cytotoxicity against endothelial cells is provoking. A possible explanation for this phenomenon may be that certain inhibiting factors that ordinarily modulate the cytotoxic activity of normal serum became adherent to the dialysis membrane, allowing the uninhibited serum to cause damage. This might be analogous to the generation of the tumoral effects observed when plasma is circulated over immobilized staphylococcal Protein A. Further studies are required to elucidate the mechanism of this phenomenon, especially in view of the theory that proposes that the defect in TTP is a deficiency of a plasma factor that normally inhibits endothelial cytotoxicity.

On the basis of the data reported here, both theories attempting to explain the pathogenesis of the disease appear plausible. If primary platelet aggregation were an initiating event, it would explain the occlusion of vessels with platelets and fibrin. This would not, however, explain the endothelial proliferation, since endothelial cells do not proliferate in response to the platelet-derived growth factor released during aggregation. However, if endothelial injury were the primary event, most of the ensuing pathology could be explained. It is generally known that following endothelial injury, platelets adhere and subsequently aggregate on the exposed collagen-rich subendothelial surface. The subsequent occlusion of vessels by platelets and fibrin would be the expected consequence. It has also been demonstrated that endothelial injury by toxic plasma factors, such as in hereditary hemolytic anemia, causes marked endothelial regeneration. Thus, both the vessel occlusion and endothelial cell proliferation of TTP may be a direct result of primary endothelial cell injury.

It is quite likely that diverse etiologies such as infection or immune complexes serve as initiators of different pathophysiologic processes (i.e., platelet aggregation or endothelial injury) leading to a common pathologic end-vessel occlusion. This would account for the disparate findings reported by investigators using plasmas from patients at different stages of the disease process. Moreover, the initiating event might operate in individuals with a genetic predisposition to stimulate antibodies capable of reacting with either platelets, endothelial cells, or both.

Seen in this light, the effectiveness of various forms of therapy for this disease can also be better understood. If platelets alone serve as antibody targets, then
pharmacologic inhibition of platelet aggregation might be sufficiently efficacious to control the disease. If, on the other hand, antibody-mediated endothelial cell destruction is the predominating process, platelet inhibitory drugs would not be helpful. In such cases, exchange transfusions or plasmapheresis would be needed to neutralize, dilute, or wash out the provoking immunoglobulin.

Evidence from the present study suggests that TTP should be viewed as an immunologically mediated syndrome with a spectrum of pathophysiolgies ranging from spontaneous platelet aggregation to massive endothelial destruction. Dissection of the prevailing mechanism in individual cases might lead to more rationally directed treatment of this devastating disease.

REFERENCES

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