Effect of Corticosteroid Therapy on the Phagocytosis of Antibody-coated Platelets by Human Leukocytes

By Robert I. Handin and Thomas P. Stossel

Glucocorticoids are often administered to patients with idiopathic thrombocytopenic purpura (ITP). Although corticosteroids may raise the level of circulating platelets in these patients, the exact mechanism by which the effect occurs is unknown. Treatment of man and various experimental animals with corticosteroids slows the disappearance of sensitized erythrocytes, platelets, and artificial particles from the circulation. The drug could diminish cell removal by influencing blood flow to the liver and spleen, the principal organs of clearance, the quantity or function of phagocytic cells in these and other organs, the amount of antibody binding to cells, or the interaction of the antibody with complement. In fact, there is evidence that corticosteroids affect many of these variables. Corticosteroid administration has been shown to
diminish hepatic blood flow, to decrease the level of circulating lymphocytes and monocytes, to retard the movement of granulocytic and monocytic cells into areas of inflammation, to decrease production of various antibodies, and to alter the binding of antibody to red blood cells.6–15

The recognition and ingestion of sensitized cells by phagocytes is a major aspect of clearance. Some investigators have reported that administration of corticosteroids to man or experimental animals inhibits the ability of various phagocytes to ingest in vitro.16–19 Others, however, have concluded that corticosteroids do not have this effect.20–25 There is also a recent report that lipid emulsions containing corticosteroids can decrease binding of antibody-coated red cells to monocytes, although ingestion has not been measured.26 The discrepancies may relate in part to the species of animals studied, the source of phagocytic cells, and the method of interacting the phagocytes with steroids. The major factor is probably the technical difficulty associated with unambiguous quantitation of ingestion by phagocytes in vitro,27 which has not been resolved in the studies cited.

We previously showed that serum from patients with ITP sensitizes platelets so that they are clearly phagocytized by autologous granulocytes.28 The phagocytosis of antibody-coated platelets by leukocytes could be quantified to provide a model for the pathogenesis of accelerated platelet clearance in ITP. In the present study, we used this system to document that corticosteroid therapy produces a phagocytic defect and that this impairment in phagocytosis correlates with the clinical response. The decreased ability of granulocytes and monocytes to recognize and ingest opsonized particles is not restricted to patients with ITP and can also be demonstrated following the administration of corticosteroids to patients with other medical disorders.

MATERIALS AND METHODS

Artificial Particles

Paraffin oil (Fisher Scientific, Pittsburgh, Pa.) containing Oil Red O dye (Allied Chemical, Pittsburgh, Pa.) was emulsified with Escherichia coli 026: B6 lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Mich.) as described previously.29 Equal volumes of these emulsions and freshly prepared human serum were incubated for 15 min at 37°C. This procedure specifically binds opsonically active complement, a fragment of C3, to the LPS-paraffin oil particles.

Preparation of Leukocytes, Platelets, and Serums

Peripheral blood leukocytes were isolated by techniques previously described whereby venous blood was anticoagulated with acid-citrate-dextrose (ACD) solution, red cells were removed by sedimentation in dextran and lysed with 0.87%, ammonium chloride, and the leukocytes were washed with 150 mM NaCl before being suspended in modified Krebs-Ringer phosphate buffer (KRP), pH 7.4.27,28 Peripheral blood mononuclear cells were prepared by centrifugation of whole blood on Ficoll-Hypaque gradients as previously described.31 Platelets were isolated from blood anticoagulated with 2.5 mM ethylenediaminetetraacetate (EDTA) by differential centrifugation, suspended in 150 mM NaCl containing 1 mM EDTA (EDTA-saline), and then incubated with test serum for 15 min at 37°C. The platelets were then sedimented, washed with EDTA-saline, and resuspended in the KRP buffer.

Rabbit antiplatelet serum was prepared by immunizing rabbits with an intravenous injection of 2 x 10⁶ platelets every other day for 14 days. The serum was then absorbed three times with an equal volume of washed type-O human red cells prior to use.
**Control Subjects and Patients**

Normal leukocytes were obtained from laboratory personnel. The diagnosis of ITP was established in six individuals with thrombocytopenia who had a normal bone marrow aspirate except for an increased number of megakaryocytes and who had no evidence of other diseases, history of drug ingestion, or splenic enlargement. The lifespan of $^{51}$Cr-labeled autologous platelets was between 2.8 and 4.8 days in three of the patients when their platelet counts were between 50,000 and 100,000/cu mm.

The corticosteroid-treated patients with nonhematologic disorders had intracranial tumor, severe asthma, or the nephrotic syndrome. These patients received either prednisone 1-2 mg/kg (Deltason; Upjohn, Kalamazoo, Mich.) or dexamethasone 0.2 mg/kg (Decadron; Merck Sharp and Dohme, West Point, Pa.). The patients with ITP had all undergone splenectomy and received cyclophosphamide (Cytoxan; Mead-Johnson, Evansville, Ind.) or azathioprine (Imuran; Burroughs-Wellcome, Triangle Park, N.C.) at various times, and all had been treated with prednisone prior to this study. In the present study, the patients were given methylprednisolone 16 mg (Medrol; Upjohn) by mouth every 4 hr for 3-5 days. Informed consent was obtained, and the study was approved by the Hospital Committee on Human Studies.

**Ingestion Rate Assays**

The uptake of paraffin oil-LPS droplets containing Oil Red O and opsonized with C3 was measured spectrophotometrically as previously described. Nitroblue tetrazolium (NBT) is reduced to NBT-formazan in phagocytic vacuoles following ingestion of a particle. Therefore the initial rate of NBT reduction during phagocytosis is an indirect reflection of the initial ingestion rate. The rate of reduction of NBT by phagocytes ingesting opsonized paraffin oil-LPS particles or platelets treated with control serum or serum containing antiplatelet antibody was assayed as described previously. In all studies, differential counts were performed on Wright-stained smears of the isolated leukocytes and results expressed per $10^7$ phagocytic cells, defined as polymorphonuclear leukocytes, bands, and monocytes. In experiments employing Ficoll-Hypaque purified mononuclear cells, the results were expressed per $10^7$ monocytes.

**RESULTS**

**Effect of High-Dose Corticosteroid Treatment on Platelet Levels in ITP Patients: Correlation with Phagocytosis of Sensitized Platelets by the Patient’s Leukocytes In Vitro**

Methylprednisolone, in high dosage, was given to six severely thrombocytopenic individuals who had previously undergone splenectomy and treatment with immunosuppressive medication, including cyclophosphamide or azathioprine as well as varied doses of corticosteroids. The complete response of a representative patient is depicted in Fig. 1. Following a 2-wk period in which there was no significant fluctuation in the platelet count, the patient was started on 16 mg methylprednisolone every 4 hr. There was a rise in platelet count that persisted for several days after corticosteroid treatment ended and then returned to pretreatment levels. A similar response was noted 1 wk later, when the patient again received methylprednisolone. Peripheral blood leukocytes obtained during corticosteroid treatment had a decreased rate of NBT reduction when incubated with allogeneic platelets that had been incubated with the patient’s heat-inactivated serum. As shown in Fig. 1, the decrease in the rate of NBT reduction correlated temporally with the periods of increased platelet count.

Similar results were obtained in the other patients studied and are summarized in Fig. 2. Following 5 days of treatment with 16 mg methylpred-
nisolone every 4 hr, their platelet counts increased three- to tenfold. Corticosteroid treatment also increased the mean level of circulating leukocytes from 5000/ to 11,000/cu mm. There was a decrease in circulating lymphocytes and monocytes and an increase in the absolute number of granulocytes.

As shown in Fig. 2, after 5 days of corticosteroid therapy the rate of NBT reduction by a patient’s washed leukocytes when incubated with allogeneic platelets sensitized with the patient’s heat-inactivated serum decreased to $\frac{1}{2}$ to $\frac{1}{3}$ of the pretreatment value. Platelets were also sensitized with serum from other ITP patients or with rabbit antiplatelet serum with identical results (data not shown). The decrease in NBT reduction by leukocytes and the elevated platelet count persisted for 3-5 days after discontinuing the corticosteroids.

In order to perform phagocytic studies on these thrombocytopenic patients, it was necessary to use platelets from unrelated normal individuals. As shown in...
INHIBITION OF PHAGOCYTOSIS BY STERIODS

Table 1. Effect of Platelet and Leukocyte Donor on NBT Reduction by Leukocytes

<table>
<thead>
<tr>
<th>Leukocytes</th>
<th>Platelets</th>
<th>Serum</th>
<th>Rate of NBT Reduction (μg Formazan/10⁷ Phagocytes/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor A</td>
<td>Donor A</td>
<td>Control</td>
<td>0.102</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ITP</td>
<td>0.280</td>
</tr>
<tr>
<td>Donor B</td>
<td>Donor A</td>
<td>Control</td>
<td>0.118</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ITP</td>
<td>0.300</td>
</tr>
</tbody>
</table>

In these experiments 2 x 10⁷ platelets from donors A and B were incubated with 100 μl heated serum from a normal individual (control serum) or a patient with ITP, washed, and incubated with 2 x 10⁶ granulocytes from donor A or B.

Table 1, there was no difference in NBT reduction when autologous or homologous platelets were sensitized with ITP serum and incubated with normal leukocytes.

Effect of High-Dose Corticosteroid Treatment on the Platelet-sensitizing Capacity of Serum From ITP Patients

Paired sera from the six patients with ITP were studied before and after treatment with corticosteroids (Table 2). The increment in rate of NBT reduction by normal leukocytes incubated with normal platelets exposed to identical quantities of serum did not decrease after corticosteroid therapy.

Effect of High-Dose Corticosteroid Therapy on Ingestion of Other Particles by Blood Leukocytes

Since analysis of ingestion by means of NBT reduction was indirect, we examined the functional properties of leukocytes from patients receiving corticosteroids with a system that measured ingestion directly and correlated the results with simultaneously determined NBT reduction rates. The results of studies on 57 normal control subjects are summarized in Fig. 3. The initial rate of ingestion of C3-coated LPS-paraffin oil particles was 0.235 ± 0.085 mg paraffin oil/10⁷ phagocytes/min (mean ± SD), and the rate was normally distributed in this control population. The rates of NBT reduction by the leukocytes ingesting in these experiments were determined simultaneously and compared to the ingestion rates. The rates of NBT reduction were expressed as ratios of formazan produced to paraffin oil ingested in order to correct for

Table 2. Effect of Corticosteroid Therapy on the Ability of Serum From Patients With ITP to Sensitize Platelets

<table>
<thead>
<tr>
<th>Duration of Treatment</th>
<th>Rate of NBT Reduction (Percentage of Controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Presteroid</td>
</tr>
<tr>
<td>6 mo</td>
<td>230</td>
</tr>
<tr>
<td>6 mo</td>
<td>210</td>
</tr>
<tr>
<td>3 mo</td>
<td>184</td>
</tr>
<tr>
<td>6 wk</td>
<td>261</td>
</tr>
<tr>
<td>3 wk</td>
<td>302</td>
</tr>
</tbody>
</table>

In these experiments 2 x 10⁷ platelets were incubated in 100 μl heated serum from five patients with ITP, washed, and then incubated with 2 x 10⁶ granulocytes. The value recorded was the percentage increase in rate of NBT reduction compared to leukocytes incubated with platelets and normal control serum. Pre- and poststeroid values were analyzed with the paired t test; there was no significant difference (p > 0.5). Control rate was set at 100%.
Fig. 3. Phagocytosis by granulocytes from corticosteroid-treated patients compared to phagocytosis by granulocytes from normal individuals. Initial rate of ingestion of serum-treated paraffin oil droplets (○); ratio of formazan produced to paraffin oil ingested (□). Control panel: mean ± 1 SD plotted for initial rate of ingestion of paraffin oil. Mean value for the two corticosteroid-treated groups was compared to that for the control group using Student’s t test (p < 0.01 in both cases). Modal ratio of formazan produced to paraffin oil ingested shown with entire range of values for 57 normal subjects tested.

changes in the rates of ingestion. The ratio of formazan to paraffin oil had an asymmetrical distribution within the normal population, with a tendency for high ratios. Thus the ratio for controls varied from 2 to 16, although 75% of the subjects studied had ratios between 4 and 6, with a modal value of 5.

As shown in Fig. 3, leukocytes from five of the six patients with ITP who received high-dose prednisone ingested paraffin oil droplets at a slower rate than normal (p < 0.01). The NBT reduction rates by the leukocytes from the treated patients were diminished in proportion to the impairment in ingestion rates. Therefore they reduced NBT normally when the value was corrected for the decreased rate of ingestion. Similar results were obtained when leukocytes were studied from nine patients receiving various corticosteroids for disorders other than ITP (Fig. 3, right-hand panel, p < 0.01). Finally, the five ITP patients’ leukocytes all ingested paraffin oil droplets at a normal rate when studied prior to corticosteroid therapy, suggesting that the observed defect was induced by the drug.

Effect of High-Dose Corticosteroid Treatment on Ingestion of Opsonized Particles by Peripheral Blood Monocytes

Similar results were obtained when purified mononuclear cell populations were studied, as shown in Table 3. The rate of uptake of LPS-paraffin oil droplets opsonized with serum was reduced in the corticosteroid-treated patients. Because of the difficulty in obtaining monocytes, it was not possible to do these studies directly on the patients with ITP. However, the data obtained with monocytes from other patients suggest that the ingestion defect induced by corticosteroids is not restricted to granulocytes.

DISCUSSION

Our studies show that the administration of corticosteroids in high doses to certain patients with ITP raised the platelet count and concomitantly inhibited phagocytosis of sensitized platelets. As shown in Table 2, corticosteroid therapy did not grossly decrease the quantity of platelet-sensitizing material in the patients’ serum or its ability to bind to platelets. However, Dixon et al. sug-
Table 3. Effect of Corticosteroid Therapy on Ingestion of C3-coated Particles by Mononuclear Phagocytes

<table>
<thead>
<tr>
<th>mg Paraffin Oil/10^7 Monocytes/min</th>
<th>Control subjects</th>
<th>Corticosteroid-treated patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.055</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>0.086</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>0.100</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>0.118</td>
<td>0.025</td>
<td></td>
</tr>
</tbody>
</table>

In these experiments 2 x 10^7 platelets were incubated with 2 x 10^6 Ficoll-Hypaque purified mononuclear cells. The rate of ingestion of C3-coated paraffin oil droplets was recorded as described in the text. The differences in rate of paraffin oil ingestion between the two groups were highly significant (p < 0.001, paired t test).

It was suggested that corticosteroid therapy may reduce the amount of antibody present on circulating platelets. The impaired phagocytic ability was not limited to patients with ITP; individuals with a variety of other disorders who required treatment with similar doses of corticosteroids developed the phagocytic defect. In addition, the lesion was not restricted to the interaction of sensitized human platelets with granulocytes. Granulocytes and monocytes isolated from corticosteroid-treated patients also ingested LPS-coated paraffin oil droplets opsonized with C3 at a reduced rate.

In earlier studies, we documented with radiochemical, phase-contrast, and electron-microscopic studies that the rate of NBT reduction by granulocytes incubated with sensitized platelets reflected ingestion of the platelets by the phagocytes. However, the rate of NBT reduction was an indirect assay for ingestion. There was a wide range of NBT reduction by normal individuals' cells in response to a standardized phagocytic stimulus because of variations in the metabolic activity of their phagocytes. This variability in the "normal" rate of NBT reduction during phagocytosis emphasized the importance of comparing a metabolic consequence of phagocytosis to an independent measure of ingestion. In the corticosteroid-treated patients studied here, the rate of ingestion of paraffin oil droplets, a direct measurement of ingestion, was decreased in parallel with the decrease in NBT reduction, indicating that the impaired NBT reduction, in fact, was due to a decrease in ingestion rates and not another metabolic effect of corticosteroids.

The cause of the corticosteroid-induced phagocytic defect is unknown. The plasma of corticosteroid-treated patients was reported to inhibit adherence of platelets to normal granulocytes. However, the data presented here suggest that an ingestion deficiency is associated with the phagocytic cell, as it can be demonstrated by incubating washed granulocytes from the corticosteroid-treated patient both with sensitized platelets from untreated individuals and with artificial particles. Corticosteroids could cause either the production or release of defective cells from the marrow or else impair the function of intrinsically normal phagocytes. In either case, the lesion could reside at a number of sites. Corticosteroids may inhibit the ability of phagocyte membranes to interact with particles opsonized with IgG or C3. Alternatively, the ingestion defect
might be related to an impairment in granulocyte adherence reportedly produced by corticosteroids, or it could involve alterations in the function of intracellular contractile proteins involved in granulocyte locomotion and ingestion. Abnormalities in either of these functions would provide a unified explanation for corticosteroid-induced impairment of granulocyte mobilization as well as ingestion.

Our observations were limited to circulating peripheral blood granulocytes and monocytes; sensitized platelets are probably removed from the circulation by mononuclear phagocytes residing in the liver and spleen. It is possible that corticosteroids might not have the same effect on these mononuclear phagocytic cells. However, studies in intact animals have consistently shown alterations in clearance within the liver and spleen using similar doses of corticosteroids. Pulmonary alveolar macrophages isolated after the systemic administration of corticosteroids were reported to have a phagocytic defect. Therefore we inferred that tissue phagocytes are similarly affected and that this is one of the mechanisms by which clearance is altered in corticosteroid-treated animals and human subjects.

The five ITP patients chosen for this study were “refractory” to the usual doses of prednisone and had persistent thrombocytopenia despite prolonged administration of immunosuppressive medication. Their sustained thrombocytopenia in the absence of therapy facilitated baseline comparisons with the effect of corticosteroid administration. The fact that they responded, although transiently, to larger doses of corticosteroids was of interest and suggested that these larger doses could be therapeutically useful in selected patients. Side effects would limit the long-term usefulness of any regimen employing very high doses of corticosteroids, although this type of treatment might be considered to control acute hemorrhage or to help prepare patients for emergency splenectomy.

ACKNOWLEDGMENT

The authors would like to acknowledge the excellent technical assistance of Howard Cohen and Vera Martin. We would also like to thank Dr. William C. Moloney and Dr. David G. Nathan for their support and encouragement.

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