Corticosteroids, Serum, and Phagocytosis: In Vitro and In Vivo Studies

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The phagocytic-bactericidal capacity (PBC) of human polymorphonuclear leukocytes (PMNs) for strains of Klebsiella (K) and of Enterococcus (E) was unaffected in vitro by the presence of 100 μg of either hydrocortisone (HC) or of methylprednisolone (MP) per ml in the medium. At higher concentrations (500 to 2,000 μg/ml) both compounds impaired PBC-K and PBC-E, but the latter was less sensitive to steroid-induced inhibition. In addition to interfering with intracellular killing of both organisms by PMNs, 2,000 μg of HC per ml also inhibited ingestion of E, although not of K. Steroid-induced inhibition of PBC-K in vitro was completely abolished by increasing the concentration of serum used as opsonin. The PBC-K of human PMNs obtained 30 min after intravenous injection of 1 g of MP was unimpaired in vitro in the presence of 10 to 90% simultaneously obtained autologous serum containing 42 μg of MP per ml. These findings suggest that short-term, high-dosage administration of MP is unlikely to produce clinically significant impairment of intraleukocytic bacterial killing.

The short-term administration of massive dosages of corticosteroids has been advocated as a therapeutic adjunct in patients with a variety of disorders including overwhelming gram-negative sepsis (4), and for such purposes as the prophylaxis of "pump lung" in patients undergoing open heart surgery (1). However, the efficacy as well as the potential risks (9) of this relatively new mode of steroid administration remain controversial (10, 16). The recent demonstration that very high concentrations (2.1 mM) of hydrocortisone (HC) may interfere with intraleukocytic killing of microorganisms in vitro (14) suggests that host defenses against bacterial infection in patients receiving short courses of massive doses of highly potent synthetic corticosteroids such as methylprednisolone (MP) might also be impaired in vivo via a similar mechanism.

We have conducted in vitro as well as in vivo experiments, and have obtained results which indicate that neither HC nor MP reduces the phagocytic and bactericidal capacity (PBC) of polymorphonuclear leukocytes (PMNs) at concentrations likely to be achieved with currently employed dosage schedules; that much higher steroid concentrations impair PBC for strains of Klebsiella (PBC-K) and Enterococcus (PBC-E) dissimilarly; and that steroid-induced inhibition of PBC-K is markedly dependent upon the concentration of serum in the test mixture.

MATERIALS AND METHODS

Bacteria. Clinically pathogenic strains of Klebsiella pneumoniae and of Enterococcus were isolated from cultures of a tracheal aspirate specimen and a urine specimen, respectively, identified by standard procedures in the Clinical Microbiology Laboratory of the Albuquerque Veterans Administration Hospital, Albuquerque, N.M., and frozen stocks of each strain were maintained at -80 C. For use in experiments, the organisms were thawed, propagated in Penassay broth (Difco), washed once in 0.5 M NaCl, and suspended in sterile distilled water. The turbidity of the suspension was adjusted to an optical density of 0.6 at a wavelength of 620 nm using a Coleman Junior spectrophotometer. They were then diluted in gel Hanks medium (GH) consisting of Hanks basic salt solution without NaHCO3 and containing 0.1% gelatin, to yield a viable count of approximately 2 × 10⁶ organisms per 0.1 ml of suspension.

Cells and opsonin. One donor (J.O.) was the source of all PMNs used, and his serum, stored at -80 C for a maximum of 3 weeks prior to use, was employed for opsonin and for studies of the effect of serum concentration on steroid-induced PBC impairment. Venous blood was collected in a heparinized (Liquaemin Sodium, Organon, Inc., 5 U/ml of blood) plastic syringe and sedimented (60 min, room temperature) in normal saline containing 6% Dextran "70" (Cutter Laboratories). The resulting supernatant of
plasma, leukocytes, and platelets was washed twice with heparinized (1 U/ml) saline, centrifuged (150 G, 5 min, room temperature) after each washing, and resuspended in GH. The number of PMNs in the suspension was determined by counting in a hemocytometer and adjusted to a final concentration of approximately 2 x 10^6 cells per 0.4 ml.

**Steroids.** HC and MP were obtained as the sodium succinate salts (Solu-Cortef and Solu-Medrol, respectively) through the courtesy of The Upjohn Co., Kalamazoo, Mich. Stock solutions were prepared in GH and stored for a maximum of 10 days at -80 C before use.

**PBC tests.** The bactericidal capacity of PMNs was determined by the method of Maaløe (12), as modified by Hirsch and Strauss (8). Tests were carried out in capped plastic tubes (12 by 75 mm) (Falcon Plastics, no. 2058); each test mixture was prepared to contain 2 x 10^4 PMNs, 2 x 10^5 bacteria, 0.1 ml of opsonin, and the desired concentration of HC or MP in a final volume of 1 ml with GH unless otherwise specified. Each steroid concentration was tested on three to five separate occasions, and control tubes omitting either opsonin or PMNs, respectively, were included in each test. Immediately after mixing, 0.001 ml was removed from each tube with a calibrated platinum loop and added to 1.0 ml of sterile distilled water to lyse the PMNs, and 0.1 ml of this suspension was pipetted into a petri dish. A 10-ml amount of warm (40 C) Pen assay agar was added, and the contents of the plates were mixed by swirling. The remaining suspension was rotated mechanically end-over-end (12 rpm, 37 C) and sampled again after 30, 60, and 120 min. The number of colonies formed by surviving viable bacteria was then counted after overnight incubation of the cultures at 37 C.

In some experiments the numbers of surviving cell-associated and non-cell-associated viable *Enterococcus* and *Klebsiella* organisms were determined (three and four individual experiments, respectively) by means of differential centrifugation techniques employing the following modifications of the above procedures. Test mixtures were prepared in 2-ml volumes, and 1-ml portions were sampled after 30 and 60 min of incubation. The total surviving colony-forming bacteria were counted as described above. The specimens were then centrifuged (150 x g, 5 min, 4 C), and 0.001 ml of the supernatant was removed for assay. Finally, each sediment fraction was reconstituted to 1 ml with GH and similarly assayed for viable bacteria.

**Statistical analyses.** Logarithms of colony counts were employed in all statistical analyses to avoid the increase in standard deviation as the mean increases (20). Analysis of variance separated effects due to concentration of steroid or serum from other effects such as the day of the experiment. Dunnett's procedure (6) was used to compare results for each steroid concentration with results for no steroid, and to compare results for serum concentrations of 30% and 60% with those for a 10% serum concentration.

**RESULTS**

**Effects of HC and MP on PBC.** The data presented in Fig. 1 to 4 indicate that the bactericidal capacity of PMNs for either *Klebsiella* or *Enterococcus* was unaffected (P > 0.05) by the presence of 100 μg of either HC or MP per ml after 60 min. However, significant impairment of PBC-K was observed at HC concentrations of 500 (P < 0.05), 1,000 (P < 0.05), and 2,000 (P < 0.01) μg/ml (Fig. 1). In contrast, PBC-E was less steroid sensitive and only the 2,000 μg/ml concentration of HC impaired PBC when *Enterococcus* was used as the test organism (Fig. 2). In nearly every instance results of comparisons after 30 min of incubation were similar to those obtained after 60 min, and after 120 min (not shown).

**FIG. 1. Effect of HC on PBC-K.** In this and all other figures, each point represents the geometric mean percentage survival calculated from data obtained in three to five individual determinations. Each P value shown in Fig. 1 to 4 refers to the significance of the difference between the mean percentage survival in the presence and in the absence of the steroid concentration (γ = micrograms per milliliter) shown. The methods employed are described in the text where abbreviations used are also defined.

**FIG. 2. Effect of HC on PBC-E.** See legend to Fig. 1.
At each of the three highest steroid concentrations tested, MP produced greater impairment of PBC-K (0.01 < \( P < 0.05 \) at 30 min; \( P < 0.01 \) at 60 min) (Fig. 3) than the same concentration of HC. However, as noted with HC, 100 \( \mu g \) of MP per ml did not diminish PBC-K significantly (\( P > 0.05 \)). PBC-E was observed to be less sensitive to the inhibiting effect of MP (Fig. 4) than was PBC-K, and only concentrations of 1,000 \( \mu g/ml \) (\( P < 0.05 \)) and 2,000 \( \mu g/ml \) (\( P < 0.05 \)) significantly inhibited PBC-E. No significant difference was detected between the PBC-E inhibition produced by HC and MP (\( P > 0.2 \)).

Experiments employing differential centrifugation (see Materials and Methods) were performed to determine whether impairment of PBC-K and PBC-E was due to a steroid-induced defect in the ability of PMNs to attach and ingest organisms or was due to defective intracellular killing of organisms, or to both. The results obtained are summarized in Table 1. Even at the highest concentration of HC employed, HC-induced impairment of PBC-K at 60 min appeared to result solely from a steroid-induced defect in intracellular killing of the organism, and over 95% of the bacteria initially present had been cleared from the supernatant. In contrast, the impairment of PBC-E by 2,000 \( \mu g \) of HC per ml appeared to reflect interference with both the attachment and intracellular killing phases of phagocytosis.

**Effect of serum concentration on steroid-induced impairment of PBC.** To simulate more closely in vitro the environment in which phagocytosis occurs in vivo, experiments were designed to test the effect on PBC of serum concentrations in excess of those customarily employed in the assay system (8). The final concentrations of bacteria, PMNs, and steroids, as well as the total volume of the reaction mixtures described above, were not altered in these experiments.

The data presented in Fig. 5 indicate that increasing the concentration of autologous serum used as opsonin to 30% (vol/vol) abolished (\( P < 0.01 \)) the inhibition of PBC-K.

### Table 1. Effect of hydrocortisone (2,000 \( \mu g/ml \)) on bactericidal capacity of polymorphonuclear leukocytes

<table>
<thead>
<tr>
<th>Determination</th>
<th>No. of surviving colony-forming bacteria/ml (( \times 10^{-4} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Klebsiella</strong></td>
</tr>
<tr>
<td>Total (30 min)</td>
<td>110</td>
</tr>
<tr>
<td>Total (60 min)</td>
<td>400 (100%)</td>
</tr>
<tr>
<td>Supernatant (60 min)</td>
<td>18 (4.5%)</td>
</tr>
<tr>
<td>Sediment (60 min)</td>
<td>190 (47.5%)</td>
</tr>
<tr>
<td>Supernatant/total</td>
<td>0.046</td>
</tr>
<tr>
<td>Supernatant/sediment</td>
<td>0.095</td>
</tr>
<tr>
<td>PBC impaired</td>
<td>+</td>
</tr>
<tr>
<td>Defective ingestion</td>
<td>-</td>
</tr>
<tr>
<td>Defective intracellular killing</td>
<td>+</td>
</tr>
</tbody>
</table>

*Methods are described in the text.

Geometric mean data from four (Klebsiella) or three (Enterococcus) individual experiments.

Polymorphonuclear leukocyte (PMN) bactericidal capacity.
induced by HC (2,000 μg/ml) and by MP (2,000 μg/ml), respectively, which was observed in the presence of 10% serum. The same concentration of serum in the absence of PMNs did not inhibit bacterial growth. In contrast to these findings, the inhibition of PBC-E induced by effective concentrations of either steroid was not reversed by 30% (Fig. 6) or even by 60% autologous serum. In other experiments, the effect of increased serum concentrations on steroid-induced inhibition of PBC was studied using Escherichia coli or group A beta-hemolytic Streptococcus strains as test organisms. The results obtained with the former resembled those described for Klebsiella, whereas those obtained with group A streptococci resembled the results when enterococci were used as test organisms.

To determine which fraction of the added serum was responsible for the reversal of steroid-induced PBC-K impairment, fresh serum was fractionated on a Sephadex G-200 column into 4S, 5S, 7S, 11S, and 19S components using standard methods (7). The resulting fractions were then compared with whole serum for their ability to reverse the inhibition of PBC-K induced by 2,000 μg of HC per ml. Each serum fraction was tested at the same final concentration calculated to be present at the corresponding concentration of whole serum. After 60 min of incubation, only 4% of the organisms survived when fractions 4S or 5S were added to the test system, thus reproducing the reversal of HC-induced inhibition of PBC-K which resulted from the addition of the corresponding concentration (60%) of whole serum. When fractions 7S, 11S, or 19S were used, bacterial survival after 60 min was 40% or greater.

**Effect of in vivo MP administration on PBC in vitro.** Despite the finding that steroid concentrations exceeding those achieved clinically (17) failed to inhibit PBC in vitro in the presence of 30 and 60% serum, an experiment was done to determine whether impaired PBC might nevertheless be demonstrable in vitro after intravenous (i.v.) injection of a very large single dose of MP. Such a circumstance might result, for example, from the production in vivo of steroid metabolites absent from the in vitro system and capable of inhibiting PBC in vitro even in the presence of relatively high serum concentrations.

Therefore, 30 min after the i.v. injection of 1 g of MP, blood for phagocytosis studies was obtained from the same subject who had provided the PMNs and serum employed in the previous studies. The 30-min time period before sampling was arbitrarily selected to allow time for in vivo production of MP metabolites, and to insure the persistence of pharmacological serum MP levels. To avoid the possible removal from the in vitro system by dilution of MP or other potentially active substances present in vivo, whole serum rather than GH was used for washing and diluting the PMNs used in this experiment. PMNs and serum obtained prior to the steroid injection were processed identically and served as a control, both with and without the addition of 1,000 μg of MP per ml to the in vitro test mixture. Each serum sample was tested in duplicate.
Impairment of PBC-K was not observed when the PMNs and serum obtained 30 min after the steroid injection were tested in vitro at serum concentrations of 10 to 90%. The concentration of MP in the serum sample obtained, kindly measured by Wayne Colburn of The Upjohn Co. by a radioimmunoassay method, was 42 μg/ml.

DISCUSSION

Therapy with corticosteroid hormones has long been recognized to predispose patients to potentially life-threatening bacterial infection (9), although the specific alterations in host defense mechanisms underlying their heightened susceptibility remain poorly understood. In vitro studies have occasionally revealed steroid-induced inhibition of bacterial ingestion of PMNs (5, 14, 19), as well as impairment of the bactericidal capacity of phagocytic PMNs for a wide variety of bacteria (14). Conversely, Allison and Adcock (2) found that phagocytosis and intracellular killing of pneumococci by PMNs obtained from healthy human donors who had received 1 to 2 mg of prednisone per kg per day for 4 days prior to testing were unimpaired. It seems pertinent to point out, however, that in these studies, as well as in the experiments reported here, the duration of steroid treatment was much shorter than that generally thought to be associated with an increased risk of clinically apparent infection. Accordingly, the relevance of these experimental data to the pathogenetic mechanisms underlying such infections is limited at best. On the other hand, such data would seem relevant to mechanisms which might enhance the susceptibility of uninfected patients to pyogenic infection by short-term, high-dose steroid therapy; such an enhanced susceptibility has not been documented, however, despite the increasingly frequent use of such therapy in a variety of clinical settings (1, 4).

Our data confirm the observations of Mandell et al. (14) that concentrations of HC which do not appear to affect ingestion of Klebsiella by human PMNs nevertheless do impair intracellular killing of these organisms in vitro. However, the concentration of HC required to produce such impairments in our studies greatly exceeds the serum concentrations produced by parenteral injection of large doses of HC to humans (17). Since the number of colony-forming bacteria present in control tubes which lacked PMNs or opsonin consistently increased two- to fourfold during the standard 60-min observation period in nearly every experiment, it seems likely that even 2,000 μg of HC per ml produced only incomplete impairment of PBC-K and PBC-E. The same high concentrations of MP were also necessary to produce impairment of PBC-K despite the fact that MP is considerably more potent than HC in many of its other biological properties (11). Inhibition of PBC-K was not observed in vitro after the addition of 100 μg of either steroid per ml. PBC-E was considerably less sensitive to both HC and MP than PBC-K. Enterococci produce H₂O₂ (13), a substance believed to participate in the intracellular killing of phagocytosed bacteria; these organisms might thus be expected to be more susceptible to intracellular destruction than Klebsiella and other bacteria which do not produce H₂O₂ (13) under conditions of impaired endogenous H₂O₂ production by PMNs. Our results are consistent with such a hypothesis.

The data presented in Table 1 also indicate that at a concentration of 2,000 μg/ml, hydrocortisone induced interference with PMN ingestion of enterococci but not of Klebsiella. They further suggest (but do not prove) that, at this concentration, HC-induced interference with H₂O₂ production by the ingested enterococci may have contributed to their intracellular survival.

The observation that the presence of additional serum in the in vitro test system completely reversed the ability of even very high concentrations of HC and MP to inhibit PBC-K reiterates the need for caution in extrapolating data obtained under relatively unphysiological in vitro test conditions to pathophysiological mechanisms prevailing in vivo or to clinical therapeutics. It seems possible that the failure of Allison and Adcock (2) to observe steroid-induced impairment of PBC for pneumococci may have been due in part to the fact that they employed an in vitro phagocytosis system containing a high concentration of autologous serum.

The possibility that the observed serum-induced reversal of steroid-induced PBC inhibition reflects binding of HC and/or MP to serum proteins is suggested by the finding that this reversal was also produced by adding increased amounts of the albumin-containing 4S and 5S serum fractions alone. Large amounts of HC and of MP are known to bind to serum albumin, although with a low affinity (18), and it seems possible that such binding might well modify the biological activity of even large concentrations of steroids in vivo as well as in vitro. Because the steroid concentrations tested were greatly in excess of the binding capacity of the corticosteroid-binding globulin (transcortin)
concentrations present in serum (15), it is unlikely that transcortin binding of HC or MP was responsible for the effect observed. Data on the percentage of protein binding of cortisol and synthetic steroids at serum concentrations comparable to those studied here are not available. However, the percentage of binding of HC was found to decrease markedly when the concentration of this steroid was increased to only 2.7 μg/ml of plasma, whereas the percentage of binding of several synthetic analogues of HC remained almost unchanged (3). Further studies of the possible role played by protein binding in mediating the effects reported here would seem warranted. The mechanisms underlying the observed differential effect of increased serum concentrations on steroid-induced inhibition of PBC-K and PBC-E, respectively, are unclear. However, this effect might possibly reflect persisting impairment of the process of ingestion of enterococci by PMNs at the high steroid levels required for PBC-E impairment. Such an effect would seem consistent with a differential sensitivity of various biological activities of steroid hormones to modification due to protein binding or possibly other serum-dependent effects.

Data pertaining to serum MP levels persisting at various times after the rapid i.v. injection of MP dosages similar to those in current clinical use, e.g., in the management of septic shock, are not available. However, in the present study, the serum MP level measured 30 min after the i.v. injection of 1 g was only 42 μg/ml. Since the half-life of HC, prednisone, and a number of other steroid hormones after i.v. injection in humans is longer than 1 h (3), this finding, together with our failure to detect any in vitro impairment of PBC after i.v. injection of 1 g in vivo, suggests that clinically significant impairment of intraleukocytic bacterial killing is unlikely to result from short-term, high-dosage MP therapy.

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W. P. Reed is a Veterans Administration Clinical Investigator.

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