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Methylprednisolone Inhibits the Alternative and Amplification Pathways of Complement

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Methylprednisolone sodium succinate limited the ability of zymosan or lipopolysaccharide to activate complement in normal serum by the alternative amplification pathways. Methylprednisolone limited B consumption in a reaction mixture which contained purified C3b, D, and B, indicating that soluble steroid directly inhibited the amplification pathway. The ability of soluble steroid to inhibit events in the alternative and amplification pathways of complement may provide a partial explanation for the effectiveness of steroids in treating gram negative septic shock.

High-dose adrenocorticosteroid therapy is used to treat a variety of immunological disorders, including gram-negative septic shock, systemic lupus erythematosus, and kidney transplant rejection (8, 24). Steroids in lower doses may function by regulating multiple events in the immunological apparatus (4, 6, 8, 23), including stabilization of membranes (1, 7, 21, 22), modulation of in vivo complement levels (2, 3), and inhibition of complement (C5a)-induced granulocyte aggregation (16, 18). Since complement is thought to be important in the pathogenesis of some of the diseases in which high-dose steroid therapy may be effective (14, 25), we felt it important to determine whether this therapy directly regulates complement activation and function. The complement system which consists of more than 18 separate proteins is activated by two pathways, a classical one (utilizing the numbered components C1, C4, and C2) and an alternative one (utilizing the lettered components B, D, and P [Properdin]). Furthermore, activation of the alternative pathway is only fully effective when there is recruitment of the positive feedback amplification loop which utilizes C3b, the major cleavage fragment of C3, as well as the alternative pathway components B, D, and P. We examined, therefore, the effect of methylprednisolone sodium succinate (MP) on the alternative amplification pathway and found that the high-dose soluble steroid was indeed inhibitory to the generation of this pathway of complement. Although steroids also inhibited later events in the complement cascade, their action at the step at which there is amplification of C3 cleavage would effectively limit complement activation and might thereby help to protect the host from the potentially lethal consequences of gram-negative septic shock.

MATERIALS AND METHODS

MP. Preservative-free MP for these studies was obtained from two sources (The Upjohn Co., Kalamazoo, Mich., and Elkins Sinn, Inc., Cherry Hill, N.J.). Both preparations of MP were examined in these studies, and there were no differences noted; in all cases both preparations were equally inhibitory.

Zymosan or lipopolysaccharide activation of serum. MP was examined for its effect on zymosan activation of serum. MP was added at 0°C to reaction tubes which contained 0.2 ml of fresh human serum, 0.2 ml of saline, and 1 mg of zymosan (ICN Nutritional Biochemicals, Cleveland, Ohio). Control tubes contained serum and saline or serum, saline, and zymosan. At time zero, the tubes were transferred to a 37°C shaking water bath, and at timed intervals, samples were removed and assayed for residual B hemolytic activity as previously described (9, 11).

MP was examined for its effect on lipopolysaccharide (LPS) activation of serum. Reaction tubes containing 0.1 ml of fresh human serum were placed at 0°C. Twenty microliters of dilutions of MP in saline or saline alone was then added. At time zero, 20 μl of LPS in saline or saline alone was added, and the tubes were transferred to a 37°C shaking water bath. At timed intervals, samples were removed and again assayed for residual B hemolytic activity (9, 11).

Effects of MP on cell-bound amplification convertase. Buffers used in these studies were half-isotonic Veronal-buffered saline (pH 7.5) containing 0.1% gelatin, 2.5% dextrose, 0.5 mM magnesium, and 0.15 mM calcium ($DGVB^{2+}$) and isotonic Veronal-buffered saline containing 0.1% gelatin and 0.04 M EDTA (0.04 M EDTA). For these studies, sensitized sheep erythrocyte intermediates (EAC4b,3b) and B, P, and D were prepared (10, 12, 13, 17). (i) Reaction tubes each containing 10^7 EAC4b,3b, 0.2 ml of $DGVB^{2+}$, and

various amounts of MP were incubated for 30 min at 30°C. The cellular intermediates were then centrifuged and washed three times in $DGVB^{2+}$ and resuspended in 0.2 ml of $DGVB^{2+}$ containing B (0.275 ng), D (100 ng), and P (700 ng). Incubation was continued for 30 min at 30°C, and then 0.3 ml of rat serum (Rockland Inc., Gilbertsville, Pa.) diluted 1:25 in 0.04 M EDTA (C-EDTA) was added to each tube. Incubation was continued for a final 60 min at 37°C. Then, 1.5 ml of saline was added to each tube; the tubes were mixed, centrifuged, and examined for lysis; and the average number of hemolytic sites per cell (Z) was calculated. Percent inhibition = Z (inhibited)/Z (noninhibited control). (ii) Reaction tubes contained 10^7 EAC4b,3b, B (10 ng), and D (100 ng) or 10^7 EAC4b,3b, B (1 ng), D (100 ng), and P (700 ng) in a total volume of 0.1 ml of $DGVB^{2+}$. At time zero, MP was added in various concentrations to the 0.1 ml of $DGVB^{2+}$, and the tubes were incubated for 30 min at 30°C, after which time 0.3 ml of C-EDTA was added, and the tubes were treated as in (i) above. (iii) The experiment in (ii) above was repeated except that the cells were centrifuged and washed three times after the 30-min incubation at 30°C but before the 60-min incubation at 37°C. After these washes, the cellular intermediates were resuspended in 0.2 ml of $DGVB^{2+}$ and 0.3 ml of C-EDTA, and the experiment was continued as in (i) and (ii) above. (iv) Reaction tubes containing 10^7 EAC4b,3b, B (10 ng), and D (100 ng) or 10^7 EAC4b,3b, B (1 ng), D (100 ng), and P (700 ng) in a total volume of 0.2 ml of $DGVB^{2+}$ were incubated for 30 min at 30°C. Then, 0.3 ml of C-EDTA containing various concentrations of MP was added to each tube, and the incubation was continued for an additional 60 min at 37°C.

Inhibition by MP of the consumption of B in a reaction mixture containing purified C3b, B, and D (26). C3b was generated from C3 as previously described (26). Reaction tubes containing 250 ng of C3b and 50 ng of D alone with incremental concentrations of MP in 150 μl of $DGVB^{2+}$ were prewarmed to 30°C, and at time zero, 550 ng of B in 50 μl of $DGVB^{2+}$ at 30°C was introduced. Incubation was continued at 30°C, and at timed intervals, 10 μl was removed from each reaction mixture, added to 0.5 ml of ice-cold $DGVB^{2+}$, and assayed for residual B (9, 11).

RESULTS

In the initial experiments, we examined the effect of MP on the ability of a solid-phase material, yeast wall (zymosan), to activate the alternative pathway of complement. At 30 min, 21, 26, 32, and 40% of initial B remained hemolytically active in the presence of MP at 0, 1, 3, and 6 mg/ml, respectively, indicating that MP in very high concentrations limited the activation of the alternative pathway in a dose-related manner.

We also examined the effect of MP on the ability of LPS, a fluid-phase substance, to activate the alternative pathway of complement. In these experiments, at 30 min, 52, 66, 70, and 81% of the initial B present in the serum was hemolytically active in the presence of MP at 0, 1.5, 3, and 6 mg/ml, respectively. Again, very high concentrations of the MP were required to limit complement activation although, at lower MP concentrations, there was significant inhibition of the ability of LPS to activate serum.

We next studied whether MP would inhibit the generation of the cell-bound amplification convertases, EAC4b,3b,Bb and EAC4b,3b,Bb,P. EAC4b,3b were (i) preincubated with MP, washed, treated with B, P, and D, and lysed with C-EDTA as a source of the terminal complement components; or (ii) incubated with MP, B, and D with and without P (which stabilizes the convertase) and lysed with C-EDTA; or (iii) incubated with MP, B, and D with and without P, washed with $DGVB^{2+}$, and lysed with C-EDTA; or (iv) incubated with B and D with and without P and lysed with C-EDTA containing various concentrations of MP (Fig. 1). Cellular intermediates preincubated with MP and then washed (i) could be lysed normally by the amplification pathway even when doses of MP as high as 6 mg/ml were used, demonstrating that MP did not bind to cell-bound C3b or to the cell directly. In contrast, when the MP at 1.5 mg/ml was introduced during the generation of the convertase (ii), it caused 88% inhibition of lysis seen with P-stabilized convertase and 83% inhibition seen with nonstabilized convertase. When these cells were washed after the generation step, but before C-EDTA was added (iii), MP at 1.5 mg/ml caused 50% inhibition of lysis seen with P-stabilized convertase and 37% inhibition seen with nonstabilized convertase. When MP at this dose was added directly to the C-EDTA step (iv), it inhibited lysis seen with P-stabilized convertase by 62% and lysis seen with nonstabilized convertase by 55%. This series of experiments showed that MP inhibited the generation or expression of the cell-bound amplification pathway convertase whether or not it was stabilized by P. These experiments have been repeated at least five times, and the variation from one experiment to another does not exceed 5%. MP also inhibited events in the terminal pathway or the ability of the cell to be lysed by complement (or both). When sodium succinate was substituted for MP as a control, no inhibition of the alternative pathway convertase generation or expression was observed.

We wished to study the effects of MP on the fluid-phase interaction of amplification pathway components to examine the mechanism of this inhibition. MP was incubated with purified C3b, B, and D. In this system, which is analogous to events in the amplification pathway, C3b binds to B (to form C3b,B) which is then cleaved by D (to form C3b,Bb). The Bb fragment may then dissociate, leaving the C3b free to bind an additional B and for the cycle to repeat (26). In the

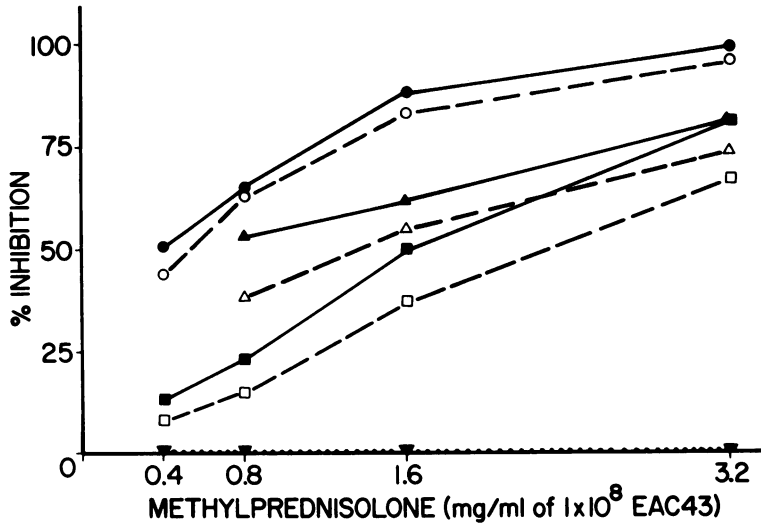

FIG. 1. Inhibition of cell-bound amplification convertase. MP failed to inhibit when preincubated with the cell-bound C3b (▼) indicating that it did not bind irreversibly to an EAC4b,3b cellular intermediate. In contrast, the MP inhibited when it was present during generation of the convertase (■, □), when it was present only during the terminal phase (▲, △), or when it was present during both generation of the convertase and during the terminal phase (●, ○). Closed symbols indicate that P was present to stabilize the convertase, and open circles indicate that P was absent. This experiment demonstrates that MP inhibited the generation of the amplification convertase and either the functioning of the terminal pathway of complement or the ability of the cell to be lysed.

presence of MP, this reaction was inhibited in a dose-related manner (Fig. 2); at 30 min, 45, 53, 64, 66, and 88% of B remained in the presence of MP at 0, 0.32, 0.62, 1.25, and 2.5 mg/ml, respectively. The MP prevented the effective utilization of B in this reaction mixture, indicating clearly that it inhibited the amplification pathway of complement. These experiments demonstrated that MP had inhibited the generation of the cell-bound convertase. MP inhibited B utilization in the fluid phase so that more residual B remained in the final hemolytic assay. The dilu-

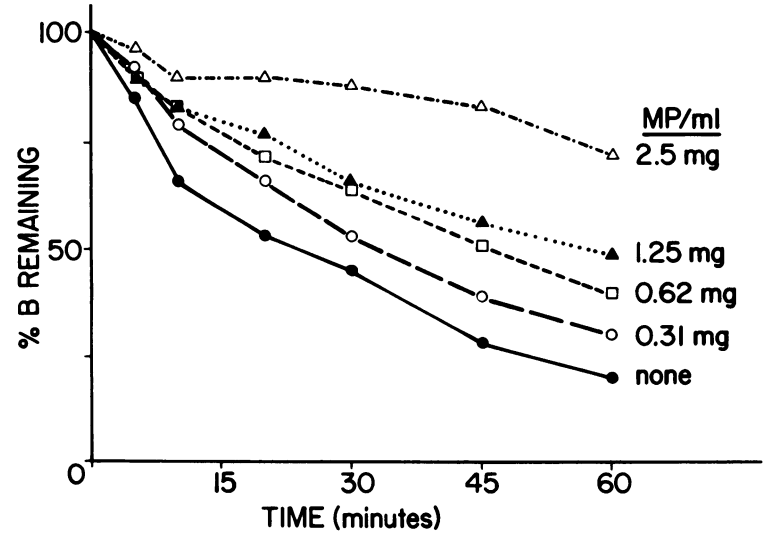

FIG. 2. MP inhibits B consumption in reaction mixture containing purified C3b, B, and D. MP at all doses inhibited fluid-phase consumption of B, indicating that MP decreased convertase formation by decreasing B utilization.

tion of the fluid-phase reaction mixture in the final hemolytic assay (1:2,000) was such that the steroid had no effect on the hemolytic intermediates as determined by control tubes.

DISCUSSION

The dose at which MP inhibited the amplification pathway was comparable to the doses of MP reported to inhibit complement-induced granulocyte aggregation (16, 18). In those studies MP was shown to regulate the ability of zymosan-activated whole plasma to cause granulocyte aggregation as measured in vitro in an aggregometer (16, 18). The high doses of MP used may be achieved in vivo when a large bolus of MP (1 to 2 g) is injected intravenously (16, 18).

It does appear from the present studies that MP inhibited both the functioning of the amplification mechanism for C3 cleavage and some later event(s) in the complement cascade (Fig. 1). This is consistent with earlier studies in which it has been shown that hydrocortisone (HC) inhibits the ability of complement to EA (15, 19). Washing these steroid-treated EA and adding fresh complement leads to lysis (19). It was found that the amount of inhibition increases with the concentration of HC and with the time and the temperature of incubation of the EA with HC (15). HC also inhibits the ability of various classical complement pathway cellular intermediates (EAC1, EAC1,4b, EAC1,4b,2a) to be lysed by the subsequent components of the classical pathway (15). These earlier studies are consistent with the present report showing that MP inhibited the ability of EAC4b,3b to be lysed. In addition, we studied the ability to lyse EAC1,4b,2a,3b,5b,6,7 by the addition of C8 and C9, which contained MP in various concentrations (data not shown). Again MP prevented lysis, suggesting an effect on C8 or C9 in the membrane attack complex or a direct action on the ability of the cell to be lysed. There was ambiguity in interpreting these studies that utilized cell-bound convertase, in differentiating between an effect of MP on the cell membrane and an effect on assembly of the alternative amplification pathway convertase. We, therefore, used the fluid-phase interaction of the purified components to study the mechanism of inhibition of the amplification pathway (Fig. 2). Indeed, in the fluid phase, MP prevented effective utilization of B in the reaction mixture that also contained C3b and D.

A variety of other substances that have antiinflammatory activities including heparin, gold salts, and complestatin (5, 20, 26) have been reported to regulate complement activation via the alternative and amplification pathways. Heparin appears to inhibit by preventing C3b and B from interacting (26), just as steroids appeared to act in the present studies. Gold sodium thiomalate also inhibits the interaction of C3b and B, again without binding to cell-bound C3b or to B. Gold does not, however, inhibit when added to the C-EDTA step (5). Complestatin, a natural product of *Streptomyces lavendulae*, appears to inhibit the alternative pathway of complement by binding and thereby inactivating B directly; when B and complestatin are incubated, B loses hemolytic activity (20). Serum levels after gold and after high-dose steroid administration in patients do approximate those at which effects have been observed in these in vitro systems. Therefore, inhibition of complement activation could be a mechanism by which these drugs exert their effects in vivo. Steroids are not usually given in such high doses. However, in gram-negative septic shock in which it has been suggested that fragments derived from complement activation may play a pivotal role in leading to disease (16, 18), these high doses are used and may limit the generation of these fragments as well as block their expression (16, 18). Although steroids may block additional steps in complement activation, a block at this critical step could help to prevent damage to the host in gram-negative septic shock. It must be noted that doses of MP that are probably not achieved in vivo were required to cause complete inhibition of the activation of complement in whole serum. Nevertheless, limited inhibition of the activation of the alternative pathway of complement may be important when coupled with the ability of MP to stabilize membranes, limit recruitment of the terminal complement pathway, and block the expression of C5a on granulocytes. These findings then may offer a partial explanation for the efficacy of high-dose MP therapy early in the treatment of patients in septic shock.

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