Corticosteroids Inhibit Complement-Induced Granulocyte Aggregation

A POSSIBLE MECHANISM FOR THEIR EFFICACY IN SHOCK STATES

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ABSTRACT Granulocyte (PMN) aggregation and embolization may underlie complement (C)-mediated organ dysfunction in such syndromes as hemodialysis neutropenia and Purtscher's ischemic retinopathy. Because of clinical and pathologic parallels, we have further suggested a role for this phenomenon in the genesis of the adult respiratory distress syndrome (ARDS). Because corticosteroids are commonly used in immune diseases, and have particularly been claimed efficacious in shock and ARDS, we tested the capability of methylprednisolone (MP), hydrocortisone (HC), and dexamethasone (DEX) to inhibit PMN aggregation.

Aggregation engendered in vitro by zymosan-activated plasma (ZAP) was inhibited by MP and HC at concentrations approximating plasma levels achieved with the large bolus (30 mg/kg i.v.) therapy advocated in shock states; DEX was almost without effect. Using intravital fluorescence microscopy, we observed PMN aggregation and embolization in the mesenteric vessels of rats given intra-arterial infusions of ZAP; this was also prevented by pretreatment with 30 mg/kg MP. Steroid inhibition of aggregation seemed not to involve disruption of receptor function, because aggregation induced by alternative agents, n-formyl-Met-Leu-Phe and the ionophore A23187, was also inhibited by MP. Moreover, corticosteroid inhibition of PMN prostaglandin synthesis is also an unlikely explanation for our results, since aspirin and ibuprofen failed to block aggregation and arachidonic acid neither effected aggregation itself nor ameliorated the steroid effect.

Our studies provide a plausible rationale for the empiric observation that high-dose corticosteroids may benefit patients with syndromes associated with microvascular leukostasis.

INTRODUCTION

Plugging of small blood vessels by leukocytes has recently received attention as a mechanism of tissue injury (1–4). Exemplified by the pulmonary vascular leukostasis of granulocytes (PMNs)[1] which characterizes the early phase of the adult respiratory distress syndrome (ARDS) (2), this phenomenon has also been indicted in the ischemic retinopathy which may occur after trauma or acute pancreatitis (Purtscher's syndrome) (3). Pulmonary leukostasis may be reproduced by the intravenous infusion of activated plasma complement (C) into animals (4), and probably underlies the C-dependent neutropenia of hemodialysis (3). We have therefore postulated a role for C-granulocyte interactions in the genesis of certain immune injuries, perhaps including ARDS (5). A possible mechanism for such leukostasis derives from our observation that PMNs aggregate when exposed to C5a in vitro (1), forming clumps which, if formed in vivo, could embolize to microvascular sites.

Portions of the work herein described were presented at the 1978 Midwest Section meeting of the American Federation for Clinical Research and at the 1978 meeting of the American Society of Hematology (1978: *Clin. Res.* **26**: 667A; 1978: *Blood.* **52**(Suppl. 1): 125).

Dr. Craddock is the recipient of a Research Career Development Award from the National Institutes of Health, 1K04 HL 00479.

Received for publication 14 December 1978 and in revised form 25 January 1979.

[1] *Abbreviations used in this paper*: AD_{50}, dose effecting 50% attenuation of aggregation wave amplitude; ARDS, adult respiratory distress syndrome; C, complement; CB, cytochalasin B; DEX, dexamethasone; DMSO, dimethylsulfoxide; FMLP, n-formyl-Met-Leu-Phe; HBSS/A, Hanks' balanced salt solution with 0.5 g/dl human albumin; HC, hydrocortisone; MP, methylprednisolone; PBS, phosphate-buffered saline; PMN(s), polymorphonuclear leukocyte(s); ZAP, zymosan-activated plasma.

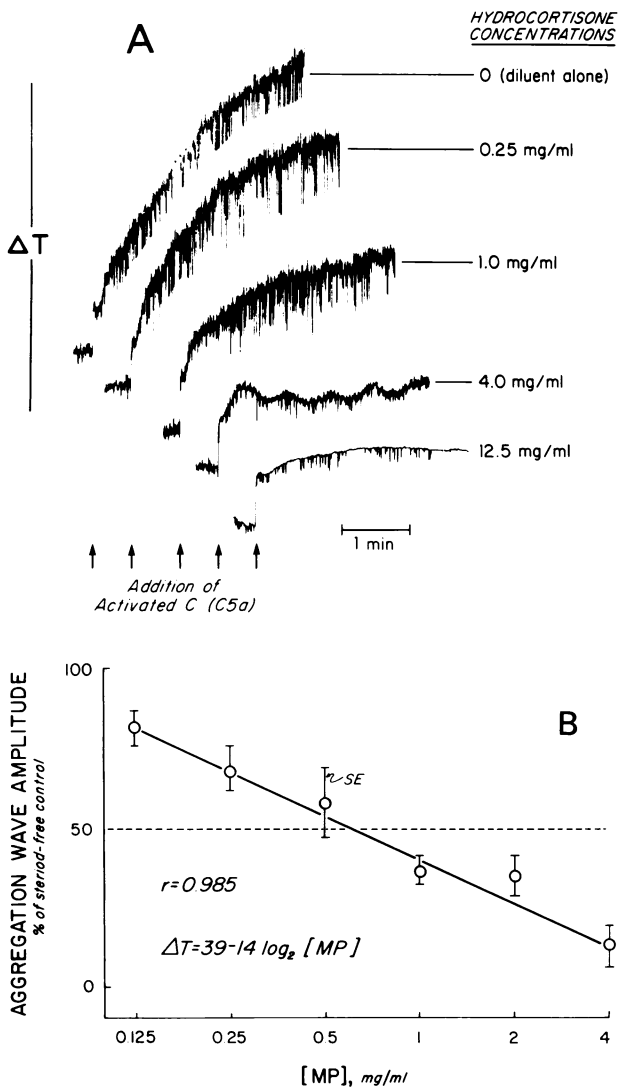

FIGURE 1 (A) Hydrocortisone (shown) or methylprednisolone inhibits PMN aggregation in response to activated C. The amplitude of the aggregation wave, increased light transmission (ΔT), is progressively dampened by increasing concentrations of corticosteroid. (B) Aggregation inhibition is a linear function of the $\log_n$ of the MP (shown) or HC concentration to which PMNs are exposed. (C) Comparison of nominally equivalent steroid doses. Nominally equivalent concentrations of corticosteroids are discordant in their inhibition of PMN aggregation; MP and HC are substantially (and equally) inhibitory, while DEX has very little effect. ZAS, zymosan-activated serum.

Two observations suggest that corticosteroids might beneficially affect this phenomenon: (*a*) in rabbits, very large doses of hydrocortisone or methylprednisolone markedly inhibit the neutropenia and leukostasis which result from infusion of cobra venom factor (6); (*b*) similar doses of these steroids favorably alter the outcome of experimental shock and ARDS (7, 8). We therefore tested the effect of corticosteroids upon the aggregation of PMNs in response to known aggregation stimuli; the results demonstrate that these agents inhibit such aggregation both in vitro and in vivo.

METHODS

Zymosan-activated plasma (ZAP) was prepared as previously described (1) and stored in 1-ml aliquots at −70°C. PMNs purified from normal human volunteers as previously described (1) were suspended ($10^4/\mu l$) in Hanks' balanced salt solution containing 0.5 g/dl human albumin (HBSS/A) (Cutter Laboratories, Berkeley, Calif.). For studies in which steroid was added after the aggregating stimulus, albumin- and Ca^{++}-free HBSS was used in place of HBSS/A, and aggregation was initiated by the addition of 0.1 vol 0.013 M $CaCl_2$.

Aggregation was quantitated as previously described (1), using a Payton 300B aggregometer (Payton Associates, Buffalo, N. Y.). Generally, to 0.4 ml PMN suspension, stirring at 900 rpm at 37°C, was added 50 μl of cytochalasin B (50 μg/ml in phosphate-buffered saline [PBS], pH 7.4), followed 1 min thereafter by 50 μl of steroid or a PBS blank; after an additional minute the aggregation stimulus was added. As in our previous studies, resulting waves were shown to represent aggregation by microscopic examination of samples fixed during the wave.

Preservative- and filler-free dexamethasone sodium phosphate (DEX) (Merck, Sharp and Dohme, West Point, Pa.), and the sodium succinates of hydrocortisone (HC) and methylprednisolone (MP) (Upjohn Co., Kalamazoo, Mich.), were provided by the manufacturers, dissolved to the (free alcohol) equivalent of 40 mg/ml, and serially diluted in PBS. Preservative- and filler-free sodium ibuprofen (Upjohn Co.) was prepared identically. Dissolved aspirin (100 mg in 0.5 ml dimethylsulfoxide [DMSO]) was diluted to 100 ml in PBS and serially rediluted in PBS containing 0.5% DMSO (vol/vol). The syn-

thetic tripeptide chemo-attractant n-formyl-Met-Leu-Phe (FMLP) (Peninsula Laboratories, Inc., San Carlos, Calif.) was prepared to 10 nM in PBS, and the ionophore A23187 (Eli Lilly and Co., Indianapolis, Ind.) was dissolved in a small volume of DMSO and diluted in PBS to a concentration of 500 μM.

Testing each steroid at least eight times, in at least four concentrations, we determined by logarithmic regression the concentration required to effect a 50% attenuation of aggregation wave amplitude (AD_{50}). Differences in AD_{50} among the agents were evaluated for statistical significance using the Mann-Whitney U test.

Female Sprague-Dawley rats were transfused with density-gradient-separated (platelet- and lymphocyte-free), fluorescein diacetate-incubated isologous granulocytes, and prepared for intravital microscopy of mesenteric capillary beds through the kind assistance of Dr. J. H. Wayland and Dr. P. D. Harris, using Wayland's previously described techniques (9). Autologous ZAP (10 ml/kg body weight) was infused intra-arterially as a bolus, either with or without immediate preinfusion of 30 mg/kg methylprednisolone.

RESULTS

Methylprednisolone or hydrocortisone, when added to a stirred suspension of PMNs, markedly impaired their aggregation response to added ZAP (Fig. 1A). Aggregation inhibition is a linear function of the $\log_n$ of the steroid concentration employed (Fig. 1B); product-moment correlation coefficients for such regression lines invariably exceeded 0.95, yielding an AD_{50} for MP of 0.63 mg/ml (1.7 mM), ±0.05 mg/ml (SEM). A similar family of curves generated for HC yielded an AD_{50} of 1.8 mg/ml (5.0 mM) ±0.49 mg/ml, whereas DEX produced very little inhibitory effect (AD_{50} unachievable; extrapolated from regression lines to approximate 32 mg/ml) (Fig. 1C). The AD_{50} value significantly differs for each steroid ($P < 0.01$).

To determine whether steroid inhibition involves interference with C5a receptor function, we employed alternative aggregating agents. From studies in chemotaxis, FMLP is known to have a receptor distinct from that for C5a (10); however, MP potently inhibits FMLP-induced PMN aggregation (Fig. 2A). Further, MP inhibited aggregation induced by the ionophore A23187 as well, doing so even when added after the ionophore (recalcification used as the aggregation trigger) (Fig. 2B).

The order in which cytochalasin B (CB) and steroid were added proved immaterial; in fact, deletion of CB (which was used to amplify aggregation response [11]) did not alter the AD_{50} values derived.

Concentrations of aspirin up to 100 μg/ml (0.57 mM), which block prostaglandin synthesis in endothelial cells and platelets (12), produced no aggregation inhibition even if PMNs were incubated with the aspirin for 30 min before the addition of ZAP; similarly, sodium ibuprofen was without effect at concentrations achieved in clinical use (data not shown).

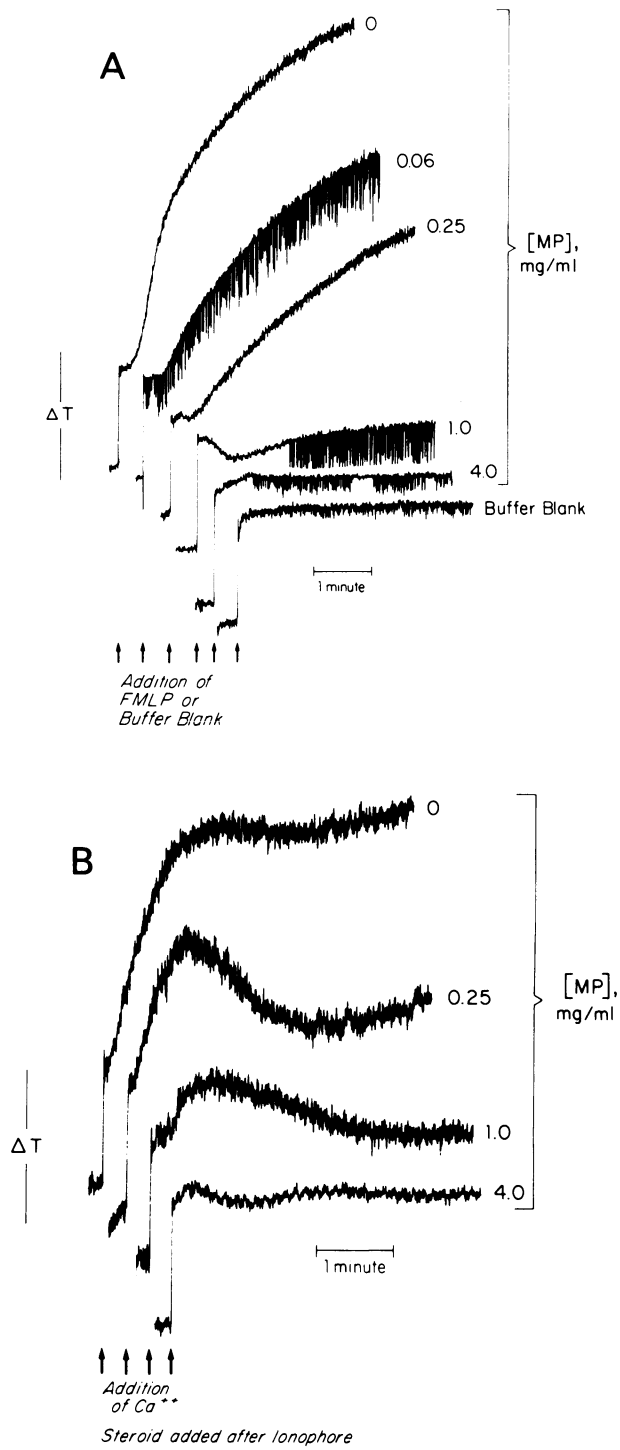

FIGURE 2 PMN aggregation engendered by alternative aggregating agents is also inhibited by methylprednisolone. (A) The synthetic chemotaxin, FMLP, is the aggregant. (B) The ionophore A23187 is the aggregant. To ensure ionophore entry into the membrane, it was incubated with the PMNs in a Ca^{++}-free medium, and recalcification then triggered aggregation.

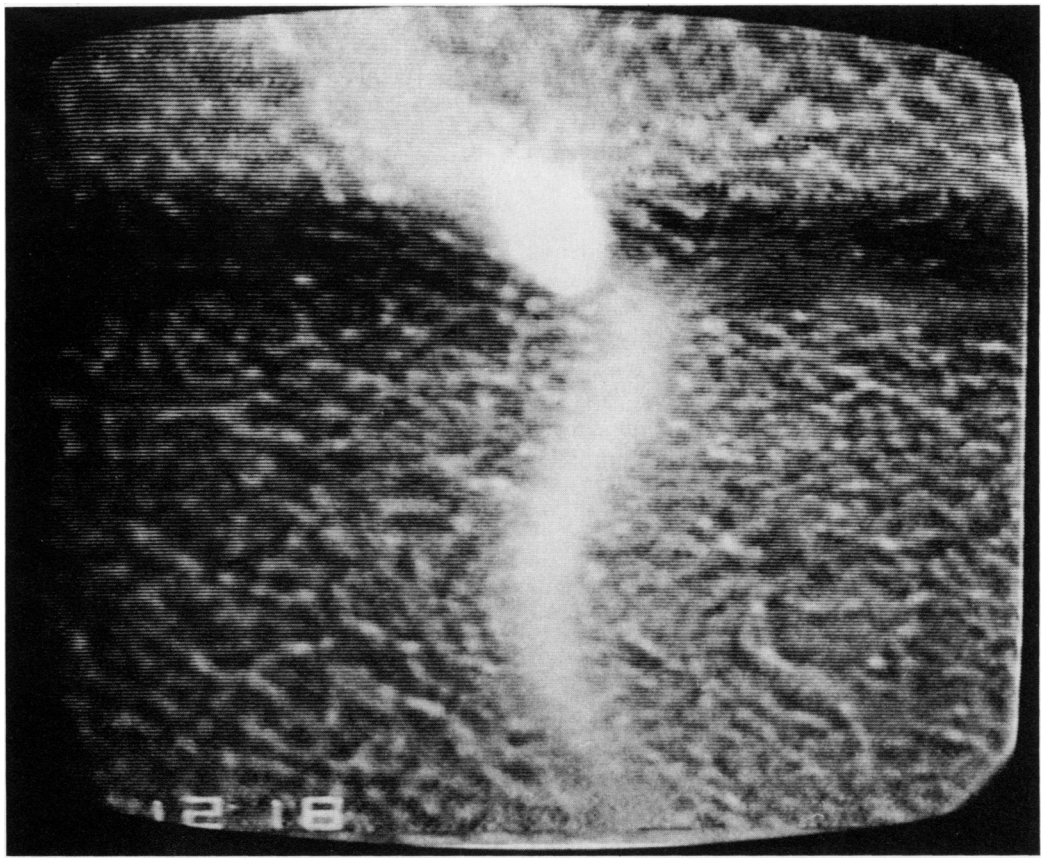

FIGURE 3 An embolizing aggregate of fluorescent granulocytes recorded in intravital microscopy of a rat mesenteric arteriole (≅50 μm diameter). Such aggregates formed and embolized to smaller vessels after intra-arterial ZAP infusion; this was blocked by MP pretreatment.

In studies to be presented in greater detail elsewhere, the intra-arterial infusion of ZAP into rats produced in their visualized mesenteric vessels immediate hypermargination of fluorescent PMNs, followed for several minutes by a meteoric display of PMN clumps, 30–70 μm in diameter (Fig. 3). These leukoemboli passed through the larger arterioles, lodging occlusively in smaller vessels downstream. Such margination, clumping, and microvascular occlusion were prevented by MP pretreatment (30 mg/kg) of the animal.

DISCUSSION

Although C-PMN interactions beneficially promote chemotaxis and the phagocytosis of opsonized microbes, inappropriate or uncontrolled interactions hold the potential for harm. Thus, in hemodialysis, activation of plasma C by dialyzer cellophane causes pulmonary vascular leukostasis, associated with lung dysfunction and peripheral neutropenia (4). Qualitatively identical consequences attend filtration leukapheresis, another extracorporeal circulation, in which nylon fibers are the C-activating polymers (13). Seeking the cause of these sudden PMN redistributions, we discovered that PMNs aggregate in response to C5a (1), which led us to propose that leukoembolization of such aggregates might be a previously-unsuspected cause of neutropenia and tissue damage. In fact, the present studies demonstrate such emboli in vivo (Fig. 3). Together with our recent observations that cultured endothelial cells are damaged by toxic oxygen radicals released by C5a-activated PMNs (14), these results suggest that C-mediated organ dysfunction may result from physical occlusion of vessels by PMNs, compounded by endothelial damage wrought by products of the stagnant, aggregated cells. If so, agents which inhibit or reverse PMN aggregation might be clinically useful.

Initial studies of patients on modest chronic prednisone doses showed no alteration in PMN aggregation response to added ZAP. But in shock states, such as

ARDS, in which microvascular leukostasis and endothelial damage are characteristic (8), modest doses of corticosteroids are ineffective, whereas extremely large doses have been claimed to be of benefit. We therefore studied water-soluble corticosteroids in concentrations calculated to exist in plasma immediately after large intravenous boluses—the usual method for their use in shock states. Indeed, such concentrations of MP (1 mg/ml) or HC (3 mg/ml), but not of DEX, inhibited by >70% the aggregation response of PMNs to activated C in vitro (Fig. 1). Moreover, intravenous MP in "shock doses" (30 mg/kg) completely prevented C-induced formation of leukoemboli in directly-visualized rat mesenteric vessels. The relative inefficacy of DEX (despite its superiority in other assays of anti-inflammatory potency) remains unexplained, although its required dephosphorylation and relatively slow cellular uptake (15) may be involved. Preliminary patient studies have shown that C5a-induced PMN aggregation is inhibited by plasmas obtained within 10 min after the administration of a 30-mg/kg bolus of MP.

We have considered several possible mechanisms for corticosteroid inhibition of PMN aggregation. First, disruption of specific membrane C receptor function seems unlikely, because steroid inhibition was comparable whether aggregation was engendered by ZAP or by other agents with receptors known to be different from that for C5a (Fig. 2). Second, because tromboxanes (the probable physiologically relevant aggregants of platelets) can be generated by PMNs (16), and because in some cell lines corticosteroids have been shown to inhibit prostaglandin synthesis by blocking arachidonate release from phospholipid (17), we wondered whether disordered PMN prostaglandin synthesis might explain our results.

Although this interpretation is attractive, our attempts to validate it were unfruitful; other prostaglandin synthesis inhibitors (aspirin and ibuprofen) were without effect and exogenous arachidonic acid, added up to a concentration of 20 μM neither promoted aggregation de novo nor overcame the corticosteroid inhibition of aggregation (data not shown).

Finally, a general quieting effect of corticosteroids upon surface membrane conformational changes of activated PMNs was sought, but futilely. Under scanning electron microscopy, MP (5 mM)-treated PMNs manifest upon exposure to ZAP the same membrane ruffling and pseudopod formation which we have observed with aggregating, steroid-free PMNs (1), despite the complete inability of such steroid-treated cells to clump.

We have preliminarily reported (5) that PMN aggregating activity (C5a) is found in the plasmas of traumatized patients who develop ARDS—an intriguing observation, since the expected in vivo correlate of such aggregating activity, microvascular leukostasis, is an early pathologic hallmark of ARDS. The present studies demonstrate that granulocyte aggregation may be inhibited both in vitro and in experimental animals by concentrations of corticosteroids approximating those claimed useful in shock states. These parallels suggest that PMN aggregation and embolization may constitute an important pathologic mechanism in ARDS; if this hypothesis is correct, then the inhibition of PMN aggregation may be a mechanism whereby high-dose corticosteroids effect their clinical benefit.

ACKNOWLEDGMENTS

The research for this paper was supported in part by National Institutes of Health grants 5T32 HL 07062, HL 19725, AM 15730, CA 15627, CA 11996, HL 16833, HL 11880, and HL 20695.

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