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ROLE OF CHEMOTACTIC FACTORS IN NEUTROPHIL ACTIVATION AFTER THERMAL INJURY IN RATS

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Abstract—Acute thermal trauma is well known to produce evidence of a "systemic inflammatory response" in vivo, as manifested by evidence of complement activation, appearance in plasma of a variety of inflammatory factors, and development of multi-organ injury. The current studies were focused on acute thermal injury of rat skin and factors responsible for accompanying activation of blood neutrophils. Acute thermal injury of rat skin resulted in a time-dependent loss of L-selectin and up-regulation of Mac-1 (CD11b/CD18) on blood neutrophils, with no changes in LFA-1 (CD11a/CD18). The loss of L-selectin was prevented by blockade of C5a but not by blockade of the α-chemokine, macrophage inflammatory protein-2 (MIP-2). C5a, the α chemokines, MIP-2 and keratinocyte-derived cytokine (KC), and platelet activating factor (PAF) contributed to up-regulation of blood neutrophil Mac-1. Blocking interventions against these mediators also blunted the degree of neutropenia developing after thermal trauma. These data suggest that activation of blood neutrophils after thermal trauma is related to the role of several chemotactic mediators. These studies may provide clues regarding factors responsible for development of the "systemic inflammatory response syndrome" after thermal injury in the experimental model employed.

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INTRODUCTION

Chemoattractants induce a range of neutrophil activation events, including polarization, chemotaxis, oxygen free-radical production, degranulation, up-regulation of neutrophil CD11b/CD18 (Mac-1) and loss of L-selectin (1–3). Furthermore, intravenous infusion of chemotactic factors induces a rapid and profound neutropenia followed by leukocytosis (4). Neutropenia may result from a cell "stiffening," leading to trapping of these cells within the narrow confines of the pulmonary microvasculature (5). Neutropenia followed by neutrophilia also occurs after thermal injury and appears to be complement-mediated (6).

Emigration of blood neutrophils into an inflammatory site requires activation of both neutrophils and vascular endothelial cells and adhesive interactions between the two cell types (7, 8). These leukocyte-endothelial adhesive events can be linked to several families of molecules: the β_2-integrins, the immunoglobulin (Ig) superfamily, and the selectin family (9, 10). L-selectin (and other selectins) appears to be important in the earliest contact between the leukocyte and the endothelium, being responsible for the "rolling" phenomenon (11). Rolling slows down the progress of leukocytes in blood vessels, particularly venules, and allows additional adhesive mechanisms to operate. The β_2-integrins, CD11a/CD18 (LFA-1) and CD11b/CD18, (Mac-1, CR3), bind to the adhesion molecule immunoglobulin family member, ICAM-1 (12, 13) which can be upregulated by exposure of the endothelium to lipopolysaccharide and cytokines (e.g., TNFα, IL-1β) (14). Under some situations this up-regulation appears to be complement-dependent (15). As neutrophils become activated by a chemoattractant, CD11b/CD18 is up-regulated and L-selectin is shed from the neutrophil membrane (16, 17). Thus, this pair of adhesion molecules appears to function in a coordinated fashion during neutrophil activation. Increased surface expression of CD11b/CD18 on circulating neutrophils after thermal injury has been described previously (18, 19), the outcome of which presumably facilitates adherence to endothelial cells. Treatment with antibodies directed against either neutrophil adhesion molecules (CD11b, CD18, L-selectin) or endothelial cells adhesion molecules (ICAM-1, E-selectin) has reduced burn-induced dermal injury as well as lung vascular injury in the experimental model (20, 21).

It is now reasonably well accepted that thermal injury induces a "systemic inflammatory response syndrome," as defined by activation of the complement system, appearance in plasma of a variety of inflammatory mediators, and activation of blood neutrophils. Activation of blood neutrophils is likely to predispose to induction of endothelial injury. The relationship between the appearance of mediators in the plasma and activation of blood neutrophils has not been established. The present study addresses the hypothesis that there is a relationship between thermal injury-induced chemotactic factor release and subsequent neutrophil activation. We used antibodies directed against C5a, keratinocyte-derived

cytokine (KC), macrophage inflammatory protein-2 (MIP-2), and platelet activating factor receptor antagonist (PAF-Ra) to define the requirements for these chemotactic factors in thermal injury-induced expression of adhesion molecules (L-selectin and β_2-integrins) on blood peripheral neutrophils of rats. Evidence is provided that after thermal injury, chemotactic factors function to cause time-dependent activation of blood peripheral neutrophils, as reflected by loss of L-selectin and up-regulation of CD11b/CD18.

MATERIALS AND METHODS

Animal Model of Thermal Injury. The rat burn model used is well established in our laboratory (6). Adult male (300 to 350 g) specific pathogen-free Long Evans rats were purchased from Charles Rivers Laboratories, Portage, Michigan. Briefly, intraperitoneal ketamine (50 mg/kg body weight) (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) and intramuscular xylazine (13 mg/kg body weight) (Loyd Laboratories, Shenandoah, Iowa) were used for continuous anesthesia and sedation (up to 4 h). After induction of anesthesia, the animals were shaved and then the lower flanks exposed to 70°C water for 30 s resulting in a deep second-degree burn. The burn was covered with sterile gauze and rats were placed in separate cages. The total body surface area burned was 28–30%, and the thermal injury was non-lethal. At various time points (as indicated) after thermal injury, blood was collected from the posterior vena cava. Then the rats were killed by excess ketamine. Control (sham burn) animals were exposed to water at 22°C. All experiments in accord with the standards in "The Guide for the Care and Use of Laboratory Animals" (DHEW Pub. No. (NIH) 78/23, revised 1978) and were supervised by veterinarians from the Unit for Laboratory Animal Care of the University of Michigan Medical School.

Leukocyte Counts. Long Evans rats (300–350 g) were kept under anesthesia for the duration of the experimental procedure. Peripheral venous blood was obtained from the tail vein using Unopette microcollection system (Becton Dickinson, Rutherford, New Jersey) at time 0 and 30 min, 1, 2, and 4 h after thermal injury and treatment (interventions). After allowing red cells to hemolyze (10 min), the white cells were counted using a hemocytometer. Numbers of polymorphonuclear neutrophils (PMN) were determined through manual differential counts (200 cells) from blood smears stained with Diff-Quick Fixative (Baxter Healthcare Corporation, McGraw Park, Illinois).

Reagents. Unless otherwise indicated, all reagents were purchased from Sigma Co. (St. Louis, Missouri).

Agents and Strategies for in Vivo Blocking of Thermal Injury-Induced Changes in Adhesion Molecules. The dosing schedules for blocking of rat C5a, PAF-R, keratinocyte derived cytokines (KC) and macrophage inflammatory protein-2 (MIP-2) were determined on the basis of recent publications (22–25). Interventions were given by an intravenous infusion.

For obtaining antibodies to rat C5a, rabbits were immunized with rat C5a obtained from zymosan-activated rat serum as described elsewhere (26). After multiple boostings, serum was obtained and rabbit IgG anti-C5a was purified by the use of a protein G sepharose column (Pharmacia Biotech AB, Uppsala, Sweden) and dialyses against PBS (22). Rats received an intravenous infusion (500 μl) of either rabbit IgG anti-C5a (1 mg/kg) or purified rabbit IgG (1 mg/kg) (positive control) in normal saline, immediately after thermal injury. PAF receptor antagonist (PAF-Ra) was kindly provided by Dr. Jeffrey S. Warren (WEB2086) (Boehringer-Ingelheim Pharmaceuticals, Inc., Ridgefield, Connecticut). Rats received an intravenous infusion (500 μl) of either PAF-Ra (50 μg/kg) (23) in normal saline or the vehicle, immediately after thermal injury. The anti-mouse KC was a

gift from Dr. Daniel G. Remick, (Department of Pathology, University of Michigan). Polyclonal antibody to recombinant mouse KC (rKC) was raised in rabbits by subcutaneous immunization with rKC in Hunters Titermax. This antibody reduces glycogen-induced peritoneal neutrophil recruitment in the rat (unpublished). Rats were intravenously infused with either 1.0 ml of a 1 : 5 rabbit immunized serum dilution or normal rabbit serum, 30 min before thermal injury. Polyclonal rabbit anti-rat MIP-2 was raised against the rat recombinant 7.9-kDa expression product (25). New Zealand White rabbits were repeatedly immunized with 1.0 mg MIP-2 emulsified in complete and subsequently incomplete Freund's adjuvant as explained elsewhere (25). Briefly, the IgG fraction of the rabbit anti-MIP-2 antiserum was obtained by chromatography on protein G-Sepharose (GammaBind G Sepharose; Pharmacia). Affinity-purified anti-MIP-2 was infused (1 mg/kg) intravenously (500 μl) into the rats, immediately after thermal injury.

Antibodies for Flow Cytometry Studies

Selectins: Antibody against rat L-selectin (HRL-3) was generated in hamsters according to previously published methods and has been shown to inhibit neutrophil binding to activated endothelium (27). The isotype matched control consisted of cells stained with hamster IgG (ImmunoPure, Pierce, Rockford, Illinois) and R-phycoerythrin goat $F(ab')_2$ anti-hamster IgG (Caltag Laboratories, San Francisco, California) labeled secondary antibody.

β_2-Integrins: Monoclonal antibody reactive with rat CD11a (WT-1), of the IgG_{2a} subclass, was generated by immunization of mice with rat splenocytes according to recently published methods (28). WT-1 has been demonstrated to recognize a leukocyte epitope of 160 to 170 kDa and did not react with the β-subunit of leukocyte function-associated antigen-1 (LFA-1) (95 to 100 kDa). This antibody has been shown to inhibit binding of stimulated lymphocytes to cultured rat high endothelial cells (28). A monoclonal antibody (1B6c) to rat CD11b was generated by immunization of BALB/c mice with rat peritoneal neutrophils and subsequent fusion of splenocytes from these mice with NS-1 myeloma cells. This antibody is described in detail elsewhere (29) and has been shown to attenuate neutrophil adhesion in vitro of rat neutrophils to rat microvascular endothelial cells. Monoclonal antibody to CD18 (WT-3) is purified IgG_1 prepared by affinity chromatography on Protein A from ascites (30). It has been shown to inhibit homotypic aggregation of PHA blasts and also blocks binding of rat lymphocytes to purified rat ICAM-1. R-Phycoerythrin-conjugated AffiniPure $F(ab')_2$ Fragment Goat anti-mouse IgG (Immunotech, Inc., Westbrook, Maine) was employed as secondary antibody. The isotype matched control consisted of cells stained with MOPC-21 (mouse IgG_1) and phycoerythrin labeled secondary antibody. The concentration of the antibodies employed was 10 μg/ml.

Measurement of Adhesion Molecule Expression by Flow Cytometry. In animals from all groups, adhesion molecule expression was determined on peripheral blood neutrophils by indirect immunofluorescence utilizing whole blood samples from the posterior vena cava (31). Blood samples were collected in heparin (10 U/ml) and were immediately fixed in 0.5% paraformaldehyde until all samples were collected. Duplicate 200 μl aliquots of blood were then incubated for 15 min at room temperature with an equal volume of 10 μg/ml of murine monoclonal antibodies specific for the desired adhesion molecule (WT-1 for rat CD11a; 1B6c for rat CD11b; WT-3 for rat CD18). An isotype matched control consisted of cells stained with MOPC-21 (mouse IgG_1). The samples were washed twice in 1.0 ml Dulbecco's phosphate-buffered saline (DPBS) and were then incubated with 100 μl of 1 : 50 dilution of the secondary antibody (phycoerythrin conjugated) for 15 min at room temperature and washed once in 1.0 ml DPBS. Red cells were lysed in 2.0 ml of FACS lysing solution (Becton Dickinson, San Jose, California) for 15 min at room temperature in the dark. The samples were then washed once in DPBS, resuspended in 1% paraformaldehyde and stored overnight at 4°C. Monoclonal antibody binding of gated neutrophil populations (identified by forward versus right angle light scatter characteristics) were measured on a FACScan Flow Cytometry System (Becton Dickinson, San Jose, California) in which 10,000 cells per determination were counted and the extent of binding was analyzed using PC-LYSIS software (Becton Dickinson). The results are expressed as percentage of controls.

Statistical Analysis. All values were expressed as mean ± *SEM* unless otherwise indicated. Statistical significance was defined as $P < 0.05$. The data were analyzed using one-way analysis of variance and differences between individual group means and control were analyzed using Student's *t*-test.

RESULTS

Expression of Neutrophil Adhesion Molecules after Thermal Injury. By the use of monoclonal antibodies, flow cytometry was employed to monitor blood neutrophil expression of L-selectin and β_2-integrins (CD11a/CD18, CD11b/CD18) after thermal injury to rat skin, as described above. Analyses were performed on whole blood samples to avoid the activating effects of leukocyte purification procedures. Levels of L-selectin and β_2-integrins were studied at 30 min, 1, 2 and 4 h after thermal injury. The isotype-matched controls consisted of cells stained with MOPC-21 (mouse IgG_1). As shown in Figure 1, within 30 min after skin burn, thermal trauma induced a reduction in L-selectin expression

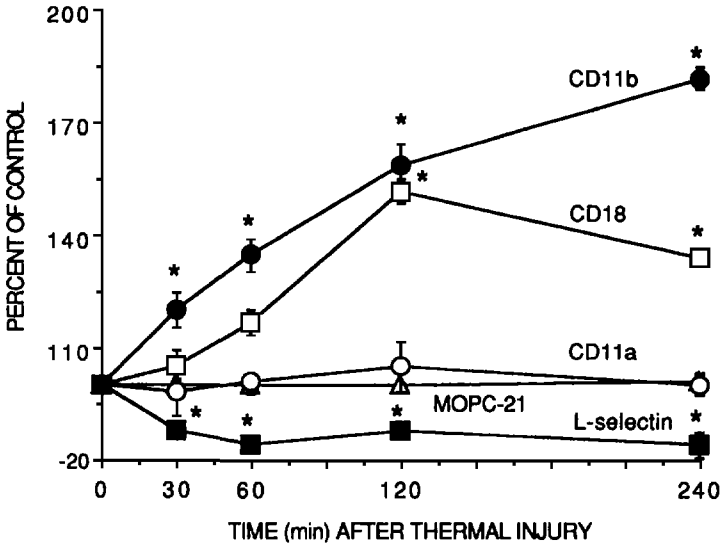

Fig. 1. Thermal injury-induced time-dependent changes of adhesion molecule (L-selectin, β_2-integrins) expression on blood neutrophils of rats, as measured by flow cytometry using monoclonal antibodies. Changes in the expression of adhesion molecules on neutrophils obtained from thermally injured rats were compared to changes observed on neutrophils obtained from control (sham burn) animals and expressed as percent of control. At least three animals per time point were used. $^*P < 0.004$ compared to control animals.

in blood neutrophils, resulting in a 15% ($P < 0.0001$) decrease (as determined by HRL-3 binding). Expression remained depressed for the entire period (4 h) of observation. A coincidental increase of 20% ($P < 0.0004$) in blood neutrophil expression of CD11b (as determined by 1B6c binding) was observed at the earliest time point (30 min). Expression of CD11b rose to 82% ($P < 0.0000002$) by 4 h. Up-regulation of neutrophil CD18 (measured by WT-3 binding) was detectable at 1 hr (17% increase) ($P < 0.004$), reaching a maximum at 2 h (52% increase, $P < 0.000003$) and slightly declining thereafter. CD18 did not return to normal levels over the 4 h period of observation. As expected, no significant change of neutrophil CD11a expression (determined by WT-1 binding) was found during these studies. Thus, thermal injury induces early and persistent neutrophil activation, as reflected by L-selectin down-regulation and up-regulation of CD11b/CD18 in the absence of changes in CD11a.

Protocols for Treatment of Experimental Animals. The finding of neutrophil down-regulation of L-selectin expression and up-regulation of CD11b/CD18 expression suggested the probability of inflammatory mediators being responsible for neutrophil activation. Chemotactic factors are known to induce similar changes in vitro (16) and it was already demonstrated in plasma the presence of neutrophil chemotactic activity, 30 min after thermal injury (6). In order to analyze the requirements of chemotactic factors on thermal injury-induced neutrophil expression of adhesion molecules, the animals were divided into 4 groups and submitted to an intravenous injection of antibodies directed to several different mediators: 500 μl of either rabbit IgG anti-C5a (1 mg/kg) or purified preimmune rabbit IgG (positive control), injected intravenously immediately after thermal injury; 500 μl of either PAF-Ra (50 μg/kg) or normal saline, injected intravenously immediately after thermal injury; 1.0 ml of rabbit serum anti-KC or normal rabbit serum, injected intravenously 30 min before thermal injury, or 500 μl of anti-MIP-2 (1 mg/kg) or normal saline, injected intravenously immediately after thermal injury. Blood was drawn from the posterior vena cava at 30 min, 1, 2 and 4 h after thermal injury and flow cytometry analysis was undertaken.

Inhibition of L-selectin Changes by Treatment with Anti-C5a and Anti-MIP-2. The intravenous injection of anti-C5a, completely prevented the loss of neutrophil L-selectin down-regulation after thermal injury (Figure 2). Why there was a statistically significant increase (16%, $P < 0.00008$) in neutrophil L-selectin at 60 min post thermal injury is not clear. Treatment with anti-MIP-2 failed to prevent the loss of neutrophil L-selectin, suggesting that, in contrast to C5a, MIP-2 does not appear to play a role in L-selectin down-regulation.

Inhibition of CD11b Expression by Blocking of C5a, KC, MIP-2 or PAF-R. Treatment with anti-C5a greatly reduced up-regulation of neutrophil CD11b expression after thermal injury at all intervals of time studied (Figure 3A). At 30 and 60 min, levels of CD11b dropped below basal levels of expres-

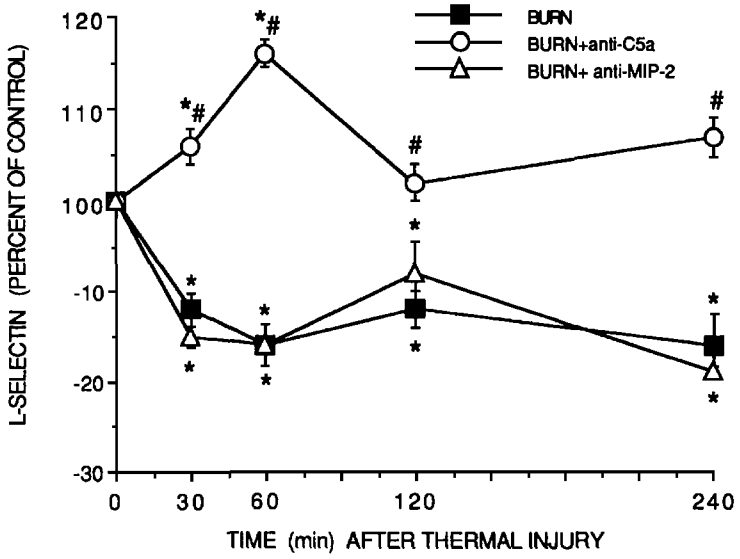

Fig. 2. Effect of blocking interventions on blood neutrophils L-selectin by intravenous infusion of anti-C5a (1 mg/kg) or anti-MIP-2 (1 mg/kg) on thermal injury-induced loss of L-selectin expression. L-selectin was determined by HRL-3 antibody binding employing flow cytometry. Changes in the expression of adhesion molecules on neutrophils obtained from thermally injured rats were compared to changes observed on neutrophils obtained from control (sham burn) animals and expressed as percent of control. At least three animals per time point were used, for each treatment group. *$P <$ 0.005 compared to control animals; #$P < 0.002$ compared to burn animals.

sion; at 2 h CD11b expression was normal, while at 4 h there was only about 20% up-regulation of CD11b in contrast to the positive controls, which, at 4 h, showed approximately 80% increase in CD11b. Thus, C5a seems to play a major role in up-regulation of neutrophil CD11b expression after thermal injury. Treatment with PAF-Ra completely abolished up-regulation of CD11b during the 4 h period of observation (Figure 3A). Treatment with either anti-KC or anti-MIP-2 failed to interfere with thermal injury-induced up-regulation of neutrophil CD11b expression at early time points (30 min and 1 h) but at 2 and 4 h either treatment reduced (40–50%, $P < 0.0009$) CD11b up-regulation (Figure 3B). Thus, C5a, PAF, KC and MIP-2 are required for full activation (defined by increased expression of CD11b) of blood neutrophils after thermal injury.

Requirements for C5a, KC, MIP-2 and PAF in Up-regulation of Neutrophil CD18. Experiments were also carried out to determine to what extent blocking of various inflammatory mediators would affect up-regulation of blood neutrophil CD18 four h after thermal injury to the skin. The data from these experiments are shown in Figure 4A, B. When rats were treated with anti-C5a, CD18

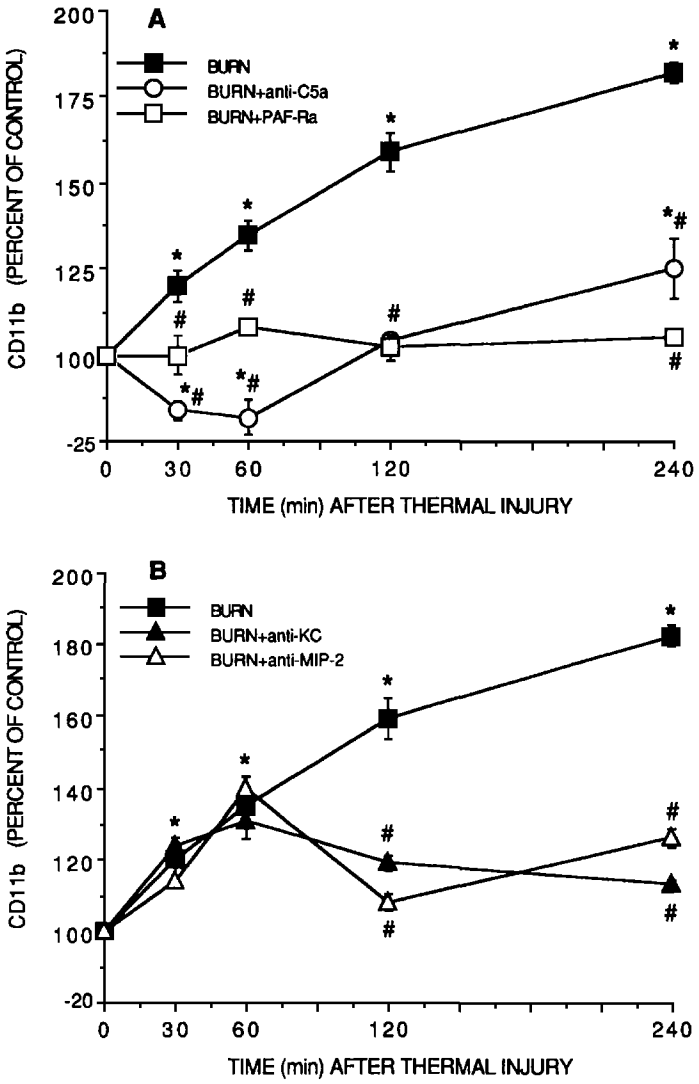

Fig. 3. Effect of blocking interventions on blood neutrophil CD11b expression by intravenous infusion of anti-C5a (1 mg/kg) or PAF-Ra (1 mg/kg) (frame A) and anti-KC (1 ml rabbit antiserum) or anti-MIP-2 (1 mg/kg) (frame B) after thermal injury. CD11b was measured by 1B6c binding using flow cytometry. Changes in the expression of adhesion molecules on neutrophils obtained from thermally injured rats were compared to changes observed on neutrophils obtained from control (sham burn) animals and expressed as percent of control. At least three animals per time point were used, for each treatment group. $*P < 0.001$ compared to control animals; $\#P < 0.005$ compared to burn animals.

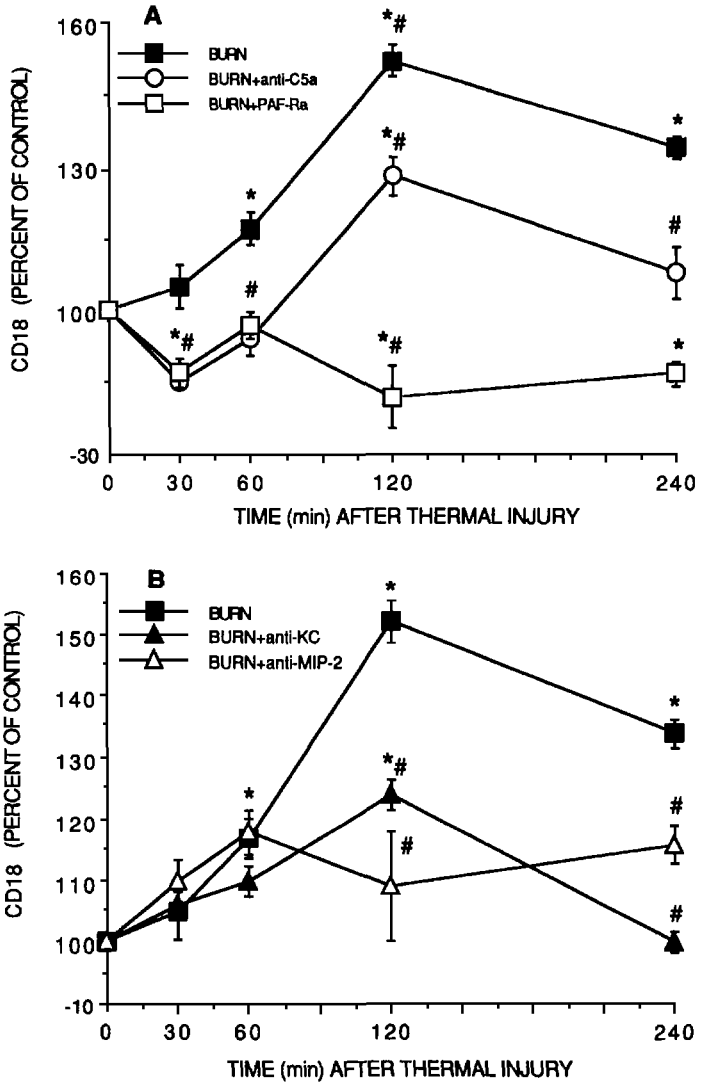

Fig. 4. Effect of blocking interventions on blood neutrophil CD18 expression by intravenous infusion of anti-C5a (1 mg/kg) or PAF-Ra (1 mg/kg) (frame A) and anti-KC (1 ml rabbit antiserum) or anti-MIP-2 (1 mg/kg) (frame B) after thermal injury. CD18 was measured by WT-3 binding using flow cytometry. Changes in the expression of adhesion molecules on neutrophils obtained from thermally injured rats were compared to changes observed on neutrophils obtained from control (sham burn) animals and expressed as percent of control. At least three animals per time point were used, for each treatment group. $*P < 0.001$ compared to control animals; $\#P < 0.009$ compared to burn animals.

Table 1. Time Course for Polymorphonuclear Leukocytes (PMN) Levels in Peripheral Blood Treatment

	PMN leukocytes (cells/mm^3)				
	0	0.5 hr	1 hr	2 hr	4 hr
Sham	3186 ± 183	2417 ± 397	2383 ± 625	2292 ± 812	2057 ± 179
Burn	3633 ± 524	859 ± 114[a,c]	2842 ± 225[c]	3992 ± 625	7358 ± 597[a,c]
Burn + anti-C5a	3550 ± 586	1433 ± 208[b,c]	1525 ± 99[b]	2261 ± 327[b,c]	6783 ± 1438[c]
Burn + PAF-Ra	3367 ± 376	1161 ± 137[c]	2301 ± 103[b,c]	3690 ± 387[c]	3893 ± 395[b]
Burn + anti-KC	3100 ± 430	1200 ± 239[c]	3450 ± 293[c]	3102 ± 246	5017 ± 420[b,c]
Burn + anti-MIP-2	2900 ± 916	1192 ± 406[c]	2050 ± 499	3022 ± 167[c]	5058 ± 480[b,c]

[a]Compared with sham ($P < 0.005$).
[b]Compared with the burn positive control group at the same period of time ($P < 0.01$).
[c]Compared with previous time point ($P < 0.01$).

levels fell below those of MOPC-21-treated rats by 15% ($P < 0.003$) at 30 min. At 60 min, CD18 levels were at baseline. At 2 and 4 h, in anti-C5a treated animals, levels of CD18 were increased by 24% ($P < 0.001$) and 26% ($P < 0.0005$), respectively, but these levels were much reduced when compared to positive controls (Figure 4A). In the case of treatment with PAF-Ra, at all time points, there was complete abolition of up-regulation of CD18 in thermally injured rats (Figure 4A). Treatment with anti-KC or anti-MIP-2 did not interfere with thermal injury-induced up-regulation of neutrophil CD18 expression at the early time points (30 min and 1 h) but treatment with anti-KC reduced CD18 expression by 28% ($P < 0.0003$) and 34% ($P < 0.00002$) at 2 and 4 h, respectively (Figure 4B). This pattern was similar to that observed at 2 and 4 h, when rats were treated with anti-MIP-2 (Figure 4B).

Effects of Interventions on Changes in Blood Neutrophil Counts. Peripheral blood was obtained before thermal injury, and at 0.5, 1, 2, and 4 h after thermal trauma. Total blood counts were determined by differential counts to determine precise neutrophil levels in blood as a result of interventions. As shown by the data in Table 1, in the sham controls a slight neutropenia developed at 30 min (a 25% fall), with blood counts stabilizing thereafter. In thermal injury animals, at 30 min, there was a 77% fall in numbers of blood neutrophils, followed by gradual return. At 4 h post burn, there was a 2-fold increase in the numbers of circulating blood neutrophils. In thermally injured animals treated with anti-C5a, the degree of neutropenia was not as severe (54% drop as compared to the thermal injury group), and there was a gradual increase at 4 h in the number of neutrophils (1.9-fold increase above time zero). Statistically, there was a significant attenuation in the degree of neutropenia in animals treated with anti-C5a when compared to thermal injury control animals. In the group treated with PAF-Ra, thermal injury lead to a 66% fall in blood neutrophils at 30 min. In this group, the treatment prevented the increase in neutrophil counts at 4 h.

When animals were treated with antibody to KC, the blood neutrophil count at 30 min fell by 61%, again indicating an attenuation in the level of neutropenia as a result of this treatment. At 4 h, blood neutrophil counts were 1.6-fold above time zero. Finally, in animals treated with antibody to MIP-2, the blood neutrophil count fell by 59% at 30 min and at 4 h the blood neutrophil count was 1.7 above the baseline. These data indicate that, of the various interventions employed, each of which reduced up-regulation of neutrophil CD11b and CD18 after thermal injury, these treatments also attenuated the degree of neutropenia developing 30 min after thermal injury. Furthermore, PAF-Ra treatment also resulted in complete prevention of the thermal injury-related granulocytosis observed at 4 h post burn.

DISCUSSION

Activation of the complement system following thermal injury has been described in the experimental setting as well as in human patients (6, 32, 33), and it has become clear that the release into the circulation of biologically active inflammatory mediators may be harmful to the host. Experimental studies revealed that thermal injury to the skin results in development of secondary injury to the lungs. Development of this injury could be related to a requirement for the complement system, since complement-depleted animals did not develop pulmonary injury as a consequence of skin burns (6). It was also observed that the complement activation resulted in the appearance in plasma of chemotactic activity (presumed to be due to C5a), an event which occurred at 30 min post burn and which was paralleled by a transient neutropenia and accumulation of neutrophils in lung capillaries. Subsequently, there was gradual development of lung microvascular injury within three to four hr after thermal injury (6). Since the development of lung injury could be related to pulmonary accumulation of blood neutrophils and their production of toxic oxygen metabolites, it was assumed that C5a might be involved in the development of secondary lung injury (6). C5a could activate blood neutrophils, causing up-regulation of neutrophil adhesion molecules, aggregation and adhesive accumulation of these cells in lung capillaries, and destruction of vascular endothelial cells by oxidants released by activated neutrophils. The recent availability of antibody to purified rat C5a and to other mediators have allowed direct experiments to be carried out in order to assess mediators responsible for activation of blood neutrophils following thermal injury.

Some of the most intriguing findings are the differences in mediator requirements for activation of blood neutrophils. C5a, but not macrophage inflammatory protein-2 (MIP-2), was required for loss of neutrophil L-selectin. C5a also appeared to be involved in post burn up-regulation of the β_2-integrin, CD11b/CD18. In this regard, it appears that platelet activating factor (PAF) has

a functional profile similar to that of C5a. Both the chemokine KC (keratinocyte-derived cytokine), which is a member of the IL-8 family of α-chemokines, and the chemokine MIP-2, also a member of the α-chemokine family, did not appear to affect blood neutrophil activation in the early phases (up to 60 min) post burn. However, these chemokines were shown to be involved in the up-regulation of CD11b/CD18 (Mac-1) at later time intervals. Treatment of experimental animals with antibody against KC or MIP-2 almost completely attenuated the up-regulation of CD11b/CD18 at four h after thermal injury. These observations may suggest that C5a and PAF play a key role in the early activation of blood neutrophils, potentially affecting other neutrophil functions such as adhesion (via CD11b/CD18) to ICAM-1 on the lung vascular endothelium, resulting in production by activated neutrophils of toxic oxygen metabolites. At later time points, KC, MIP-2 and PAF also seem to play important roles in neutrophil activation post burn.

It is unclear why L-selectin downregulation was prevented by blockade of C5a but not by blockade of MIP-2, while blockade of either molecule prevented up-regulation of CD11b/CD18. It may be that MIP-2 is generated in rather small amounts in the first 2 h after thermal injury, whereas substantial production of C5a had occurred by this time point. Cell activation of neutrophils resulting in shedding of L-selectin is thought to be due to events leading to cathepsin activation and cleavage of L-selectin near its outer transmembrane region (34). Up-regulation of CD11b/CD18 appears to be due to fusion of secondary cytoplasmic granules to the cell membrane, resulting in the appearance of Mac-1 at the outer surface of the neutrophil cell membrane. Accordingly, it might be predicted that rat MIP-2, in contrast to C5a, does not cause activation of neutrophil cathepsin which results in cleavage of the N-terminal region of L-selectin (34). One of the other interesting facets of these data is the fact that, under the conditions employed, blockade of the C5a, KC, MIP-2 or PAF-R resulted in nearly total suppression in up-regulation of Mac-1 at 2 and 4 h after thermal injury. This suggests that these mediators may function in a sequential manner, each one depending on another to bring about the full biological response. In other words, it may be that C5a is necessary for chemokine and PAF expression and that blockade of either one may interrupt steps that lead in vivo to up-regulation of Mac-1. It is difficult at present, to determine whether sequential engagement of these mediators is occurring or if engagement is occurring simultaneously.

Systemic neutrophil activation by C5a has been suggested to occur in thermally injured patients as well. Increased content of the complement receptor CR3 (Mac-1) has also been observed on blood neutrophils during the first 5 days after thermal injury (18). It was suggested that this may be C5a-mediated because of systemic complement activation and suppressed in vitro responses of blood neutrophils to zymosan(complement)-activated serum (18). Similar observations and suggestions were made by Nelson and coworkers who noticed up-regulation of CR3 (19) and down-regulation of C5a receptors on blood neutrophils of

thermally injured patients (35). Engagement of adhesion molecules other than L-selectin and β_2-integrins has also been observed in thermally injured animals. Vascular injury at the dermal burn site in rats was shown to require involvement of Mac-1, ICAM-1 and E- and L-selectin (20). In the same study, development of lung injury secondary to skin burns in rats was demonstrated to require Mac-1 as well as LFA-1 (CD11a/CD18). Other adhesion molecules involved in secondary lung microvascular injury included ICAM-1 and E- and L-selectin. Whether development of acute lung microvascular injury following skin burns is related to chemoattractant-mediated activation of blood neutrophils remains to be elucidated. Because of their immediate activating effects on blood neutrophils, C5a and PAF may seem particularly strong candidates. That increased blood neutrophil adhesiveness can result in accumulation of neutrophils in lungs has recently been suggested and can also be deducted from our current observations of circulating neutrophils during burn injury. Fried and colleagues studied changes in blood neutrophil adhesiveness and aggregation in thermally injured mice (36). Increases in adhesiveness and aggregation of blood leukocytes were observed within 1 h after thermal injury only to further increase up to 6 h and then to gradually decrease and subside by 5 days after thermal injury. The degree of leukocyte adhesiveness and aggregation correlated with the degree of pulmonary leukostasis. We have noticed that all chemoattractants under study were involved in the development of neutropenia, suggesting that these chemotactic mediators are also involved, at least in the pulmonary accumulation of blood neutrophils.

Based on the current studies, thermal injury results in activation of blood neutrophils, the activation process appearing to be due to the action of chemotactic mediators. The present data demonstrate that all chemoattractants studied (C5a, PAF, KC, MIP-2) were involved in activation of blood neutrophils following thermal injury, with C5a and PAF appearing to play a crucial role in early activation events. These data indicate that neutrophil activation in the experimental model is due to effects of C5a, chemokines and the lipid mediator PAF. This would be consistent with the concept that thermal injury incites a "systemic inflammatory response syndrome," resulting in the appearance of several mediators that cause inappropriate activation of blood neutrophils, setting the stage for multi-organ injury.

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