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MEDIATORS OF MICROVASCULAR INJURY IN DERMAL BURN WOUNDS

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Abstract—In previous studies we have demonstrated that second-degree thermal injury of skin in rats leads to secondary effects, such as systemic complement activation, C5a-mediated activation of blood neutrophils, their adhesion-molecule-guided accumulation in lung capillaries and the development of acute pulmonary injury, largely caused by neutrophil-derived toxic oxygen metabolites. In the dermal burn wound, however, pathophysiologic events are less well understood. The injury is fully developed at four hours post-burn. To further elucidate the pathogenesis of the "late phase" dermal vascular damage, rats were depleted of neutrophils or complement by pretreatment with rabbit antibody against rat neutrophils or with cobra venom factor, respectively. In other experiments, rats were treated with blocking antibodies to IL-6, IL-1, and TNFα immediately following thermal burning or were pretreated with hydroxyl radical scavengers (dimethyl sulfoxide, dimethyl thiourea). Extravasation of ^{125}I-labeled bovine serum albumin into the burned skin was studied, as well as, skin myeloperoxidase levels. The studies revealed that, like in secondary lung injury, neutrophils and toxic oxygen metabolites, are required for full development of microvascular injury. In contrast, however, development of dermal vascular damage in thermally injured rats was not affected by complement depletion. Our data suggest that the development of microvascular injury in the dermal burn wound is complement-independent, involves the pro-inflammatory cytokines IL-1, TNFα and IL-6, and may result from reactive oxygen metabolites generated by neutrophils accumulating in the burn wound.

INTRODUCTION

There have been significant advances in recent years in the treatment and care of burn patients. Therapies such as topical antibiotic administration, early debridement and grafting of affected areas and vast improvements in intensive care management have contributed to a marked increase in survival rates (1). Despite this progress, the pathophysiology of the burn wound is still not fully understood. Second-degree dermal burn wounds provide an interesting point of investigation as this type of trauma results in extensive tissue damage not only locally, but

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in distant organs, as well. The systemic effects occur in cases with extensive thermal trauma, but can be seen even in burn injuries covering only one quarter of the total body surface area (TBSA). In a second-degree thermal injury model in rats involving 25–30% TBSA, secondary lung injury has been observed. Till, et al. (1983) described systemic complement activation, as detected by reductions in hemolytic activity of individual complement components (C3, C4, C6) and crossed immunoelectrophoresis analysis of the conversion of serum C3 (2). Chemotaxis assays showed C5a-mediated activation of blood neutrophils to occur, as well. Secondary accumulation of polymorphonuclear leukocytes (PMN) has been shown to occur in lung tissues, followed by development of acute pulmonary injury, related to production of neutrophil-derived toxic oxygen metabolites (2). Employing the same animal model of dermal burn injury, PMN influx into lung tissues was reduced substantially (up to 77%) using antibodies to adhesion molecules (LFA-1, Mac-1, ICAM-1, E and L-selectin) (3).

The process of local edema formation in the burn wound appears to be more complex, as it involves both the direct effect of heat and the consequences of inflammatory mediators locally generated in response to the thermal insult. It has been shown that under conditions of limited thermal, chemical or physical trauma, two waves of increased vascular permeability occur in the skin (4). The "early phase" of increased permeability—approximately one hour after thermally injury—was shown to develop as a result of complement activation with anaphylatoxin release and mast cell secretion of histamine. This lead to an enhancement of xanthine oxidase activity and increased production of oxygen radicals, damaging endothelial cells (5). This one hour injury has been shown to be neutrophil independent (6).

The pathophysiology of "late phase" dermal microvascular injury (at approximately four hours post-burn) is less well understood. The delay in onset of this injury would indicate that a series of elaborate events must take place before maximal tissue damage is achieved. Recent data from our group suggest that, in contrast to the "early phase" edema formation, blood neutrophils are involved in the pathogenesis of the "late phase" injury. Since antibodies to neutrophils, as well as, to E- and L-selectin and ICAM-1 were shown to effectively reduce vascular leakage in the dermal burn wound at four hours post-burn (3), we sought to clarify the mechanism for neutrophil recruitment and infiltration by focusing on the upstream inflammatory mediators that may regulate these events. The current studies were designed to elucidate the role of the cytokines IL-6, IL-1 and TNFα, as well as complement, and to further clarify the role of neutrophils in the development of microvascular injury in the second-degree, "late phase" dermal burn wound. Here, we present evidence which demonstrates that the four hour burn injury is complement independent, involves the proinflammatory cytokines IL-6, IL-1 and TNFα and may result from the tissue-damaging effects of neutrophil-derived reactive oxygen species.

MATERIALS AND METHODS

Animal Model of Thermal Injury

The experimental burn model used in the present study has been described previously (2, 6, 7). Adult male, specific pathogen-free Long-Evans rats (300–350 g, Harlan Sprague-Dawley, Indianapolis, Indiana) were used in all experiments. Ketamine hydrochloride (100 mg/kg body weight) (Fort Dodge Laboratories, Fort Dodge, Iowa) and xylazine (13 mg/kg body weight) (Bayer Corporation, Shawnee Mission, Kansas) were administered intraperitoneally and intramuscularly, respectively, throughout the experiment. This ensured that the animals were properly anesthetized for the entire procedure, from the induction of the burn injury to the time of sacrifice. The skin over the lumbrosacral and dorsal flank areas was shaved and exposed to 70°C water for 30 s. This resulted in a deep second-degree skin burn involving 25 to 30% of the total body surface area. Animals were sacrificed at 4 h by cervical dislocation. Control animals were exposed to 22°C water. All experiments were in accord with the standards in *The Guide for the Care and Use of Laboratory Animals*, and were supervised by veterinarians from the Unit for Laboratory and Animal Care of the University of Michigan Medical School.

Measurement of Microvascular Injury. Local microvascular injury was assessed by measurement of extravasation of radiolabeled bovine serum albumin (^{125}I-BSA) into the burned skin. Immediately prior to thermal injury, burn or sham-treated animals received an intravenous injection of 0.5 μCi of ^{125}I-BSA in 0.5 ml sterile phosphate buffered saline (PBS). Using a template, four uniform skin samples, each one square inch in size, were excised from the burned area on each animal. For calculations of the permeability index, the amount of radioactivity (^{125}I-BSA) in skin biopsies was compared to the amount of radioactivity present in 1.0 ml of blood obtained from the inferior vena cava at the time of sacrifice (4 h).

Measurement of Skin Myeloperoxidase (MPO) Content. Local accumulation of neutrophils was assessed by measurement of myeloperoxidase in skin biopsies. Animals received burn or sham treatment as described above. At time of sacrifice (4 h), four 4 mm punch biopsies from standardized areas of the wound were taken from each animal and instantly frozen in liquid nitrogen. The biopsies were homogenized in 500 μl of PBS pH 7.4, containing 0.1% Tween 20, sonicated on ice and insoluble material removed by centrifugation at 3000 rpm for 10 min. 5 μl of tissue extract (PBS pH 7.4 and 0.1% Tween 20) were incubated with 100 μl of 2, 2′-Azino-di-[3-ethylbenzthiozoline sulfonate (6)] diammonium salt solution (ATBS substrate) (Boehringer Mannheim, BIOCHEMICA, Germany) and the maximum velocity of the substrate/MPO chromogenic reaction (Vmax) measured by monitoring the 96 well low-protein binding flat bottom plates (Corning Glass Works, Corning, New York) at 405 nm over a two minute period (BioTek Elx808 microplate reader) (BIO-TEK Instruments, INC., Winooski, Vermont). Kinetic calculations were performed using KC3 software (BIO-TEK Instruments, Inc.). MPO concentrations in samples were determined using a standard curve of purified MPO (CALBIOCHEM, San Diego, California). MPO values are reported as units of activity/biopsy.

Interventional Studies

Cytokine Blockade. Irrelevant IgG antibody, anti-mouse IL-6 polyclonal antibody, and anti-rat IL-1β monoclonal antibody were obtained from R&D Systems, Minneapolis, Minnesota. Anti-rat TNFα polyclonal antibody were purchased from PeproTech, INC., Rocky Hill, New Jersey. In

each case, antibodies were given in a total amount of 500 μg per animal, in 0.5 ml sterile PBS, administered intravenously in two equal doses at 30 and 120 min post-burn.

Complement Depletion. Cobra venom factor (CVF) was purified from crude lyophilized cobra venom (Naja naja kaouthia) (Sigma Chemical Company, St. Louis, Missouri) by ion exchange chromatography and gel filtration (8). Complement depletion was achieved by serial intraperitoneal injections of 4 X 20 units CVF in 12 h intervals, resulting in undetectable levels of serum hemolytic complement activity (CH50 Assay). The experiments were performed 12 h after the final injection of CVF.

C5a Blockade. Isolation of polyclonal antibody to C5a was performed as described by Mulligan, et al. (9). Briefly, animals were immunized with rat C5a. Obtained serum was IgG purified by acid elution of Sepharose G beads (Pharmacia Biotech AB, Uppsala, Sweden), followed by extensive dialysis against PBS. Characterization of the anti-rat C5a antibody was performed by immunoprecipitation and Western blot analysis showing a single band at 14 kDa. This antibody was given in a total amount of 400 μg per animal, administered intravenously in two equal doses at 30 and 120 min post-burn.

Neutrophil Depletion. Neutrophil depletion was induced by the intraperitoneal injection of 1.0 ml of rabbit antiserum to rat PMN (Accurate, Westbury, New York) 16 h prior to the experiment. This procedure reduced neutrophil counts in peripheral blood by >90 percent.

Hydroxyl Radical Scavenger Administration. Dimethyl thiourea (DMTU) (Sigma Chemical Co.) (1000 mg/kg body weight) in 1.0 ml sterile PBS was injected intraperitoneally 10 min prior to thermal injury. Dimethyl sulfoxide (DMSO) (Sigma Chemical Co.) (500 μg) in 1.0 ml sterile PBS was injected intraperitoneally 10 min prior to thermal injury. The effectiveness of the chosen concentrations of scavengers was demonstrated in earlier studies (7).

Statistical Analysis. Data sets were analyzed using one-way ANOVA. Individual group means were compared with the Tukey multiple comparison test. All values were expressed as mean $\pm$ *SEM*. Significance was assigned where $P < 0.05$. For percentage change between groups, values obtained from negative controls were subtracted from each data point. Statistical analysis was performed using SigmaStat 2.0 (Jandel Scientific Software, San Rafael, California).

RESULTS

Protective Effects of Neutrophil Depletion in Dermal Burn Injury. Neutropenia was achieved by intraperitoneal injection of antiserum to rat PMN. Extravasation of ^{125}I-bovine serum albumin into the skin 4 h after thermal trauma was used to measure tissue injury. The results of neutropenia on the development of increased vascular permeability in the skin are shown in Figure 1. Negative controls had a permeability index of 0.049 $\pm$ 0.039. Neutrophil depletion was associated with a 58% ($P < 0.001$) attenuation of the dermal vascular permeability four hours after thermal injury (permeability index of 0.405 $\pm$ 0.038 in neutrophil depleted rats versus an index of 0.898 $\pm$ 0.039 in nontreated rats). Thus, availability of PMNs seems to be required for the full development of dermal microvascular injury four hours after thermal trauma.

Failure of Complement Depletion to Protect Against Dermal Microvascular Injury. Complement depletion was induced with CVF as described above. Extravasation of ^{125}I-bovine serum albumin into the skin four hours after ther-

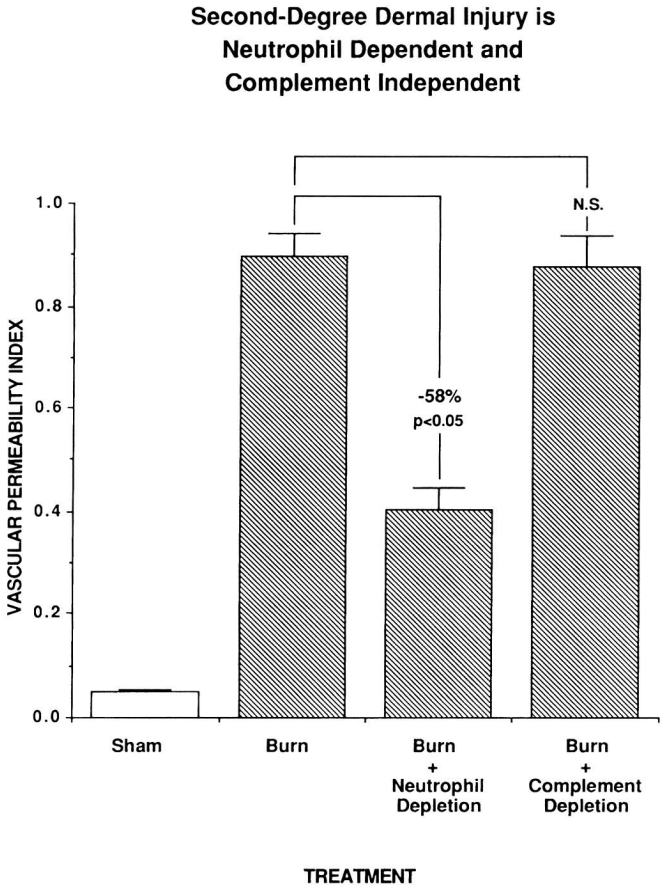

Fig. 1. Effects of complement depletion and neutrophil depletion on dermal vascular injury 4 h after thermal trauma to skin as measured by leakage of ^{125}I-labeled bovine serum albumin. For each vertical bar, $N = 4$.

mal trauma was used to assess tissue injury. The results of complement depletion on the development of increased vascular permeability in the skin are shown in Figure 1. Complement depleted rats had a permeability index of 0.88 ± 0.065 versus an index of 0.898 ± 0.039 in non-treated rats (PN.S). Thus, complement depletion was not associated with a significant reduction in dermal vascular permeability compared with positive controls.

Failure of C5a Blockade to Protect Against Dermal Microvascular Injury. In the presence of antibody to C5a, vascular permeability index was calculated. Anti-C5a-treated rats had an index of 0.89 ± 0.054, as compared with an index of

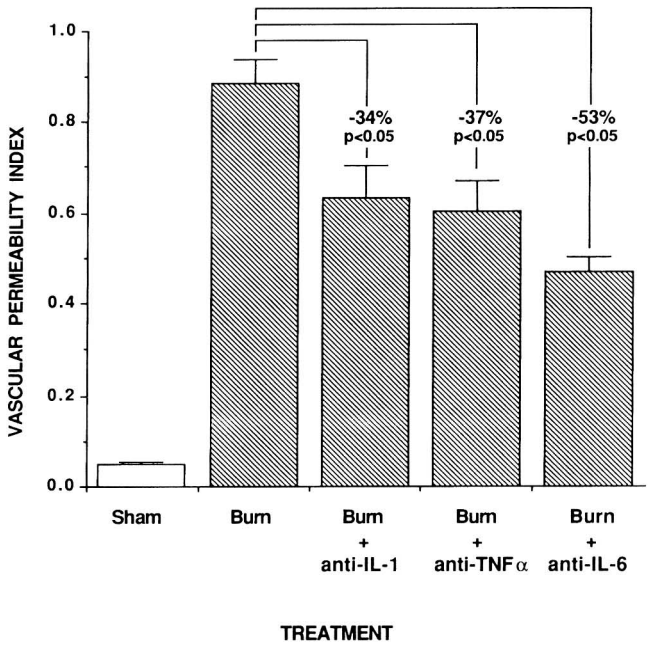

Fig. 2. Effects of cytokine blockade on dermal vascular injury 4 h after thermal trauma to skin as measured by leakage of ^{125}I-labeled bovine serum albumin. For each vertical bar, $n = 5$ or higher.

and 0.89 ± 0.047 in positive controls (PN.S.). C5a blockade was not associated with a significant reduction in dermal vascular permeability compared with positive controls. Thus, C5a does not seem to be required for dermal microvascular injury four h after thermal trauma, providing further evidence that development of the "late phase" burn wound is complement independent.

Protective Effects of Cytokine Blockade Against Dermal Microvascular Injury. Protection against increased vascular permeability in skin 4 h after thermal injury was evaluated through the use of blocking antibodies to cytokines. Vascular permeability index was calculated as described above. The negative control skin permeability index was 0.049 ± 0.002; this value increased to 0.885 ± 0.048 in positive controls, as shown in Figure 2. Treatment with anti-IL-6 was

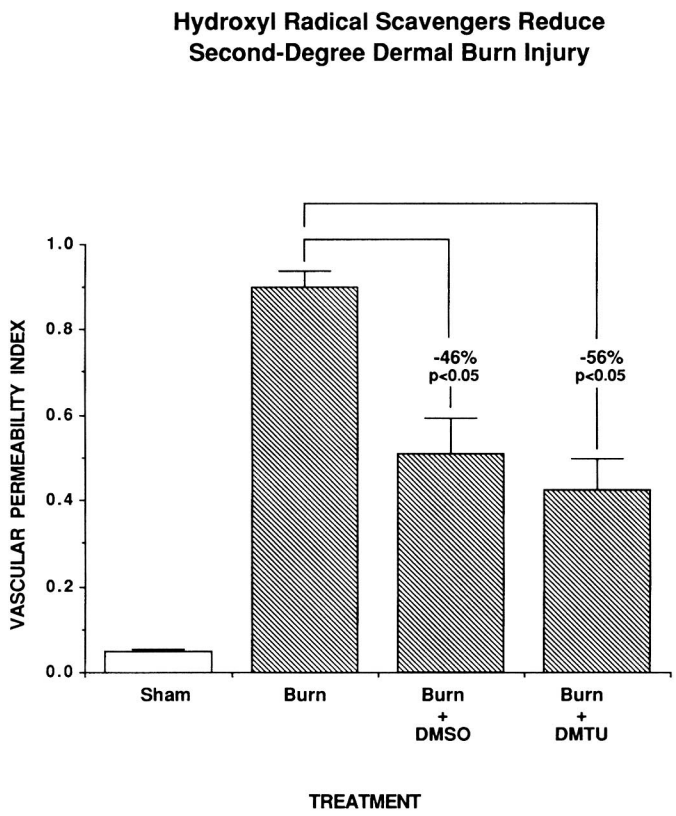

Fig. 3. Effects of hydroxyl radical scavengers on dermal vascular injury 4 h after thermal trauma to skin as measured by leakage of ^{125}I-labeled bovine serum albumin. For each vertical bar, $n = 4$.

associated with a 53% ($P < 0.001$) reduction in dermal vascular permeability to 0.47 ± 0.031. Treatment with antibody to IL-1 resulted in a decrease of dermal vascular permeability by 34% ($P < 0.001$) to a value of 0.63 ± 0.068. Similarly, animals treated with anti-TNFα had a mean dermal vascular permeability index of 0.60 ± 0.068, which was 37% ($P < 0.001$) lower than the positive control group. Thus, dermal microvascular injury at four hours after thermal trauma requires the cytokines IL-6, IL-1 and TNFα for full development.

Protective Effects of Hydroxyl Radical Scavengers in the Skin Burn Wound. The effects of hydroxyl radical scavengers on vascular permeability in skin 4 h after thermal injury was assessed by determination of vascular permeability index. The data are shown in Figure 3. Treatment with dimethyl thiourea (DMTU) was associated with a 56% ($P < 0.001$) reduction in vascular perme-

Table 1. Protection Against Neutrophil Influx into Thermally Injured Skin

Treatment	No. of animals	MPO Value (x ± SEM)	Significance (P values)	Change (%)[a]
None	4	23.81 ± 0.20	<0.001	
anti-IL-6	4	8.70 ± 0.81	<0.001	−67
anti-IL-1	4	9.34 ± 1.60	<0.001	−64
anti-TNFα	4	7.41 ± 0.11	<0.001	−73
anti-PMN	4	6.94 ± 0.33	<0.001	−75

[a]For percentage change between groups, values obtained from negative controls were subtracted from each data point.

ability as compared to the positive controls (0.426 ± 0.070 for DMTU treated rats, 0.898 ± 0.039 in non-treated rats). Treatment with dimethyl sulfoxide (DMSO) resulted in a 46% ($P < 0.001$) reduction in dermal vascular permeability (0.508 ± 0.081 with DMSO treatment, 0.898 ± 0.039 without). Thus, blockade of hydroxyl radicals results in a marked protective effect on dermal microvascular injury four hours after thermal trauma, indicating an important role for these radicals in the "late phase" local burn injury.

Role of PMN in the Dermal Burn Wound. Skin samples from injured animals treated with irrelevant antibodies or specific antibodies directed against the cytokines IL-6, IL-1 and TNFα or against rat PMNs were collected and assessed for MPO activity as a measure of tissue accumulation of neutrophils. Negative control animals showed values of MPO content of 1.31 ± 0.019, increasing to 23.8 ± 0.20 in samples from positive controls. The results of interventional studies of vascular leakage (Figure 1) were noticeably similar to PMN accumulation in these treatment groups (Table 1). Anti-IL-6 treated animals showed a MPO value of 8.7 ± 1.6 which represents a 67% ($P < 0.001$) reduction in PMN accumulation. Anti-IL-1-treated animals showed a 64% ($P < 0.001$) decrease in MPO value to 9.3 ± 3.2. Anti-TNFα-treated animals displayed an MPO value of 7.4 ± 0.23, a reduction of 73% ($P < 0.001$) as compared to positive controls. As would be expected, neutrophil depletion resulted in a 75% ($P < 0.001$) reduction in MPO value to 6.9 ± 0.66. Therefore, blockade of IL-6, IL-1 and TNFα greatly reduced tissue MPO activity, demonstrating a clear requirement for these cytokines in neutrophil accumulation during the "late phase" dermal burn injury.

DISCUSSION

Our data indicate that development of the late phase of microvascular leakage (four h post-burn) in thermally injured rat skin requires the pro-inflammatory

cytokines IL-6, IL-1 and TNFα. The ability of blocking antibodies to each of the aforementioned cytokines to attenuate both vascular injury and polymorphonuclear leukocyte (PMN) influx into the burn wound suggests that these cytokines represent upstream mediators in this inflammatory process. These cytokines may affect vascular endothelial cells (EC), which are known to actively participate in the development of inflammatory reactions by controlling fluid leakage and promoting adhesion and activation of leukocytes, or target PMN. With regard to IL-1 and TNFα, there is a large body of evidence detailing the ability of these cytokines to activate EC to synthesize and express adhesion molecules (10–14). The adhesion molecules ICAM-1 and E- and L-selectin have previously been shown to be required for full development of vascular injury in the "late phase" edema formation of thermally injured rat skin (3). In addition, IL-1 and TNFα have been shown to induce cellular production of IL-6 (15, 16).

There are several ways in which IL-6 may exert pro-inflammatory effects on increased vascular permeability and PMN influx in acute local thermal injury. There is in vitro evidence that IL-6 increases endothelial permeability by rearranging actin filaments and by changing the shape of endothelial cells (17). Biffl, et al., have demonstrated that with platelet-activating factor, IL-6 potentiates PMN priming and delays PMN apoptosis (18, 19), both effects which contribute to PMN-mediated tissue damage. Furthermore, Mullen et al. demonstrated that IL-6 is capable of interacting synergistically with TNFα to augment the effect of TNFα on PMN phagocytosis and superoxide production in vitro (20). The authors hypothesized IL-6 to be a more distal mediator of the cytokine cascade, which may modulate an inflammatory response to trauma initiated by other, more proximal cytokines. This supports our data which show IL-6 blockade to be the most effective of the cytokine interventions in reducing dermal vascular injury after thermal trauma. Lastly, expression of ICAM-1 on myocytes is induced by IL-6 in a cardiac ischemia/reperfusion injury model (21). Given the established role of ICAM-1 and E- and L-selectin in the development of "late phase" edema in thermally injured skin, it is possible that IL-6 may exert a similar effect on EC in the pathogenesis of burns, resulting in increased PMN recruitment and infiltration into injured tissues.

PMN depletion resulted in a significant decrease in both vascular leakage and PMN accumulation in skin at 4 h. This is in contrast to the 1 h burn wound for which PMN involvement was not required (7). The delay in burn wound edema formation may be explained by the time necessary for the cytokine cascade to cause expression of adhesion molecules in the injured skin and activate PMNs. The notion that increased tissue damage then results from PMN-derived reactive oxygen species is supported by our data showing the ability of hydroxyl radical scavengers in these experiments to reduce vascular injury. The fact that PMN depletion, or any of the other interventions, was only able to result in a maximum reduction of approximately 60% in vascular leakage is most likely

due to the possibility that a large percentage of injury and cell death (approximately 40%) is directly heat-related. A similar phenomenon was observed in the assay of skin MPO content.

It is known that the burn model utilized in these experiments produces systemic complement activation that initiates a series of events leading to the "early phase" edema formation in the burned skin. Interestingly, neither complement depletion nor C5a blockade was able to attenuate local vascular permeability at four hours. In this case, the effects of cytokines and PMNs may be able to produce maximal injury even in the absence of complement. Another possibility is that the role of complement is fulfilled in the early phase, whereas the late phase is more dependent on the ensuing inflammatory reaction. Despite the fact that our data does not support a role for complement in the "late phase" burn wound, a complete lack of involvement of complement components cannot be stated. Recently, studies have revealed local complement production in numerous and varied tissues in an ischemia/reperfusion injury in rabbit (22). Complement proteins may be present in thermally injured tissue and contributing to the development of the 4 h injury, but were not effectively blocked by our antibody to C5a or were able to be produced in significant amounts in skin, though serum complement levels were undetectable following treatment with CVF. Administered in one dose immediately prior to induction or thermal trauma, 400 μg of anti-C5a was able to slightly, though not significantly, reduce vascular leakage at four hours (data not shown).

We therefore conclude that the development of the "late phase" dermal vascular injury following thermal trauma to the skin is largely mediated by the pro-inflammatory actions of cytokines, in particular IL-6. IL-6 may modulate the effects of TNFα and IL-1, resulting in EC expression of E- and L-selectin and ICAM-1. IL-6 also may act directly on EC to alter cell structure and/or promote upregulation of adhesion molecules. PMN-generated reactive oxygen species appear to be responsible for the local tissue damage. Complement does not appear to play a significant role, if any, in the pathogenesis of the "late phase" second-degree burn wound.

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