(U) Immunorodulation by agents which inhibit suppressor cells in injured mice

John F. Hansbrough, M.D.

We have completed extensive studies on immune downregulation after defined injury in mice. We employed a standard model of musculoskeletal injury, crush injury of the hind limb coupled with immediate limb amputation, which has been shown by other groups to lead to immune suppression and to the generation of suppressor cells. We compared immunity in these animals to immunity in animals receiving a moderate-sized (25% BSA) full-thickness burn injury.

We found that limb trauma led to slight decreases in lymphocyte proliferation, but lymphocyte activation steps as measured by surface antigen expression (IL-2R, Ia) on helper and suppressor lymphocyte subpopulations were minimally altered. Specific antibody responses were studied, using both primary and secondary immunization to sheep erythrocytes (T-dependent) and to endotoxin (T-independent); these responses were not altered after limb trauma. Neutrophil function, measured by the stimulated...
oxidative burst, was slightly decreased after limb trauma. In contrast, these three
immune functions were markedly depressed after 25% BSA burn injury.

Further studies showed that implantation of burned tissue or devitalized,
nonburned skin or other tissue into normal mice via a subcutaneous pocket recreated
all of the immune dysfunctions seen after burn injury. We suspect therefore that
immune dysfunction after injury may result from an amplified local host response to
devitalized tissue; this may involve elements of the inflammatory response.

Further work has sought to identify potential mediators of immune dysregulation
after injury or stress. We studied the effects of catecholamines, prostaglandins, and
histamines, since all three mediators are increased after injury. We incubated these
mediators in vitro with lymphocytes or with neutrophils, prior to functional analysis
(lymphocyte proliferation or neutrophil oxidative burst). Beta-adrenergic agents,
PGE2, and a histamine-2 agonist all produced downregulation of both lymphocyte and
neutrophil functions. These studies suggest that devitalized tissue contributes to
immune downregulation after injury, and that circulating or tissue mediators which
have been showed to be elevated after injury may contribute to immune dysfunction
after injury.
Severe injury appears to frequently lead to a state of immune suppression which undoubtedly contributes to subsequent infections. In addition, general stress associated with or in the absence of injury may also contribute to immune suppression, with predisposition to infections. During the three years of this contract we have intensively studied immunologic parameters in mice following a variety of injuries, to determine the types of immune dysfunctions which occur after injury, and the mechanisms of immune suppression to the type of injury. We have also attempted to identify possible mediators of immune suppression which might play roles in immunologic events which occur following injury. The identification of mediators of immune suppression, some of which may be blocked by antagonists of various types, will hopefully lead in the future to effective pharmacologic therapy which will prevent immune downregulation after injury.

II. PROGRESS REPORT

A. Lymphocyte subsets

There has been hope that the numbers and phenotypes, and the ratios, of various subsets of peripheral lymphocytes might reflect the degree of immune function of the host. Therefore, peripheral blood splenic lymphocyte subsets after various types of murine injury were exhaustively studied for their expression of surface antigens in the first year of this project. Lymphocyte preparations were labeled with monoclonal antibodies specific for helper (L3T4), suppressor/cytotoxic (Lyt2), IL-2 receptor (IL-2R), and Ia surface antigens. There is evidence that IL-2R and Ia antigen expression may reflect subpopulations of immunoregulatory or activated T lymphocytes which may play important roles in immunity. Two-color flow cytometry was used for analysis so that the latter two antigens could be studied on both helper/inducer and suppressor/cytotoxic populations.

Spleen and peripheral blood lymphocytes were used for phenotype analysis on various days after simple musculoskeletal trauma. This trauma model was developed by Mannich et al. at Harvard, and has been shown to be accompanied by a degree of immune suppression with mediation of the suppression at least partly via a "suppressor" monocyte populations. Some of the results from our laboratory are shown in Figure 1. While there were transient depressions in some of the cell types, no substantial changes in the helper/suppressor ratio were seen. These results are markedly in contrast to changes we had seen in burned mice, where we found prolonged depressions in numbers of lymphocytes.
bearing all of the surface antigens under study.

B. Lymphocyte proliferation and activation

Lymphocyte activation is accompanied by marked changes in the expression of surface antigens, and much recent evidence indicates that some of these surface antigens play important roles in the activation and signaling processes of lymphocytes. We therefore studied patterns of surface antigen expression after musculoskeletal trauma. In Figure 2 we compare these changes seen after musculoskeletal trauma to changes seen after murine burn injury. Minimal changes in surface antigen expression, compared to cells from control mice, were seen after nonburn trauma. In addition, minimal decreases were seen in the proliferation potential of splenic lymphocytes from nonburn, injured mice, while lymphocytes from burned mice had severely impaired proliferation. These studies suggest that musculoskeletal trauma, while shown to be immune suppressive in several laboratories, does not present the severe immunosuppressive challenge to T cell function seen by burn injury.

C. Neutrophil function after injury

In a series of experiments we studied neutrophil (PMN) phagocytosis and oxidative burst after injury, using assays which we carefully developed utilizing flow cytometry. The phagocytosis assay utilized measurement of the uptake of fluorescein-labeled microorganisms, with quantitation of positive cells by flow cytometry. In developing this assay we showed that uptake absolutely required the presence of serum or immunoglobulin, confirming the clinical relevance of the assay; in contrast, previous assays which utilized fluoresceinated beads do not require opsonization. The assay for oxidative burst used the dye DCFH-DA, which is nonfluorescent but which becomes fluorescent when it reacts with intracellular hydrogen peroxide. Again, cell fluorescence is quantitated by flow cytometry. We studied these two cellular functions on days 1, 5 and 10 following injury.

We studied phagocytosis by using fluorescein-labeled S. aureus, P. aeruginosa, and S. faecalis incubated with PMNs. Uptake of the particles was then measured using flow cytometry. We found no reproducible depression in phagocytosis after burn injury or musculoskeletal trauma.

However, burn injury produced severe depression of the stimulated oxidative burst by 5 days postinjury which persisted for several weeks after injury. In contrast, musculoskeletal trauma resulted in an initial depression in the oxidative burst on day 1 postinjury; the defect was however reversed at 5 days after injury (Figure 3).

In further experiments we implanted various tissues into otherwise normal mice, to see if tissue effects would influence immune functions. This can be easily done by creating a dorsal pocket to receive the tissue; the pocket can then be simply closed using a suture. The neutrophil oxidative burst was then followed on various days.

Implantation of burned or unburned skin or liver tissue produced marked depression of the oxidative burst, measured 10 days later (Figure 4). These studies suggest that PMN dysfunction after injury may be due in part to the presence of devitalized tissue. Since our model of musculoskeletal trauma in the mouse includes the amputation of the limb following the crush injury and fracture, this may explain the lack of severe immune depression occurring
On this trauma model. To our surprise, the depression after tissue implantation was not dependent only on burned skin, but occurred with other devitalized tissues (skin, liver) which were devitalized by freezing and thawing. These experiments suggest that the devitalized tissue may release various immunosuppressive mediators, although it is equally likely that the host response to dead tissue may produce an immunosuppressive effect. For example, inflammatory neutrophils or macrophages may release mediators such as histamines, prostaglandins, etc, and subsequent infection may produce endotoxins. Various potential mediators were therefore studied in the following experiments.

D. Effect of mediators on lymphocyte proliferation and on the PMN oxidative burst

We have now tested for the effects of multiple mediators on the functions of both lymphocytes and neutrophils from mice. We selected for study mediators which might play a role in contributing to immune dysfunction after injury. These mediators included:

1. Histamine-2 agonist (Impromidine)
2. Catecholamines (epinephrine, isoproterenol)
3. Endotoxin
4. Prostaglandin E2

In a long series of experiments, we incubated these mediators in various concentrations with lymphocytes at the time of initiation of culture, with stimulation by ConA or PHA; tritiated thymidine incorporation was then measured 48 hours later to measure proliferation. In some experiments we measured the activation of lymphocytes by measuring surface antigen expression (L3T4, Lyt2, IL-2R, and Ia) using monoclonal antibodies and two-color flow cytometry.

In separate experiments we incubated the mediators with neutrophils (PMNs) harvested from the peritoneal cavity; the oxidative burst of the PMNs was then measured using the dye DCFH-DA and flow cytometry.

Some of these results are shown in Figures 5, 6, 7 and 8. In summary, we can make the following observations:

1. Histamine-2 suppresses lymphocyte proliferation and the PMN oxidative burst
2. Beta-adrenergic drugs suppress the PMN oxidative burst but not lymphocyte proliferation
3. Endotoxin had no effect on PMN or lymphocyte functions
4. PGE_2 suppressed lymphocyte proliferation and the PMN oxidative burst

E. ANTIBODY PRODUCTION AND CLEARANCE AFTER INJURY

Using a modified Jerne-plaque technique, we measured specific antibody production after burn injury and musculoskeletal trauma in the mouse. We measured the primary and secondary responses to sheep erythrocytes (SRBCs) and also to endotoxin (LPS), by coupling the LPS to SRBCs.

We found that burn injury resulted in a tremendous augmentation in the specific antibody responses to both SRBC and LPS. Musculoskeletal injury, in contrast, did not produce an augmented response.
We measured IgG clearance in injured mice by injecting human IgG and measuring subsequent levels of hIgG by an ELIZA technique. Burn injury resulted in greatly enhanced clearance, while musculoskeletal trauma did not result in altered clearance.

III. IMPLICATIONS

These studies have two important conclusions at this time:

A. Immunosuppression after injury may be related in part to the presence of, and/or reaction of the host toward, devitalized tissues.

B. Various inflammatory and stress mediators, including histamines, catecholamines, and prostaglandins, may play important roles in contributing to immunosuppression after injury.

It will be important in the future to further study these phenomena. Preliminary experiments in our laboratory are directed at studying the ability of antagonists of the various mediators named above to improve immune function after stress and injury.

IV. INVENTIONS: NONE

V. PUBLICATIONS RELATED TO THIS CONTRACT: