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IMMUNOMODULATION BY DRUGS WHICH INHIBIT SUPPRESSOR CELL  
FUNCTION IN TRAUMATIZED MICE(U) CALIFORNIA UNIV MEDICAL  
CENTER SAN DIEGO J F HASBROUGH 21 SEP 87

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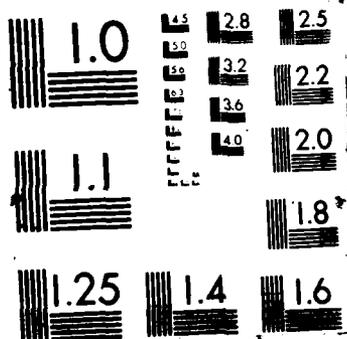
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<p>The purpose of this project is to determine if the immunosuppression which occurs after severe injury can be reversed or prevented by pharmacologic agents. The first step has been to set up defined measures of immunity which reflect postinjury immunosuppression in the mouse. These assays have now been set up; they include T cell subset analysis (blood, lymph node, spleen), T cell mitogenic response with assay for surface antigen expression, neutrophil (PMN) phagocytosis, PMN chemiluminescence, and serum opsonic activity. We are now ready to begin to study the effect of various drugs on these immune functions, which are all inhibited in the injury model. The drugs to be studied in the 3rd year include prostaglandin blockers, histamine-2 antagonists, and cyclophosphamide, an alkylating agent which in low-dose has been shown to inhibit suppressor cell proliferation.</p>			
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ANNUAL REPORT, ONR CONTRACT N-00014-85-K-0652  
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SUBMITTED SEPT 20, 1987

**PRINCIPAL INVESTIGATOR:** John F. Hansbrough, M.D.

**CONTRACTOR:** Univ. of California at San Diego Medical Center

**CONTRACT TITLE:** Immunomodulation in Traumatized Mice

**ORIGINAL START DATE:** 1 SEPT 1985

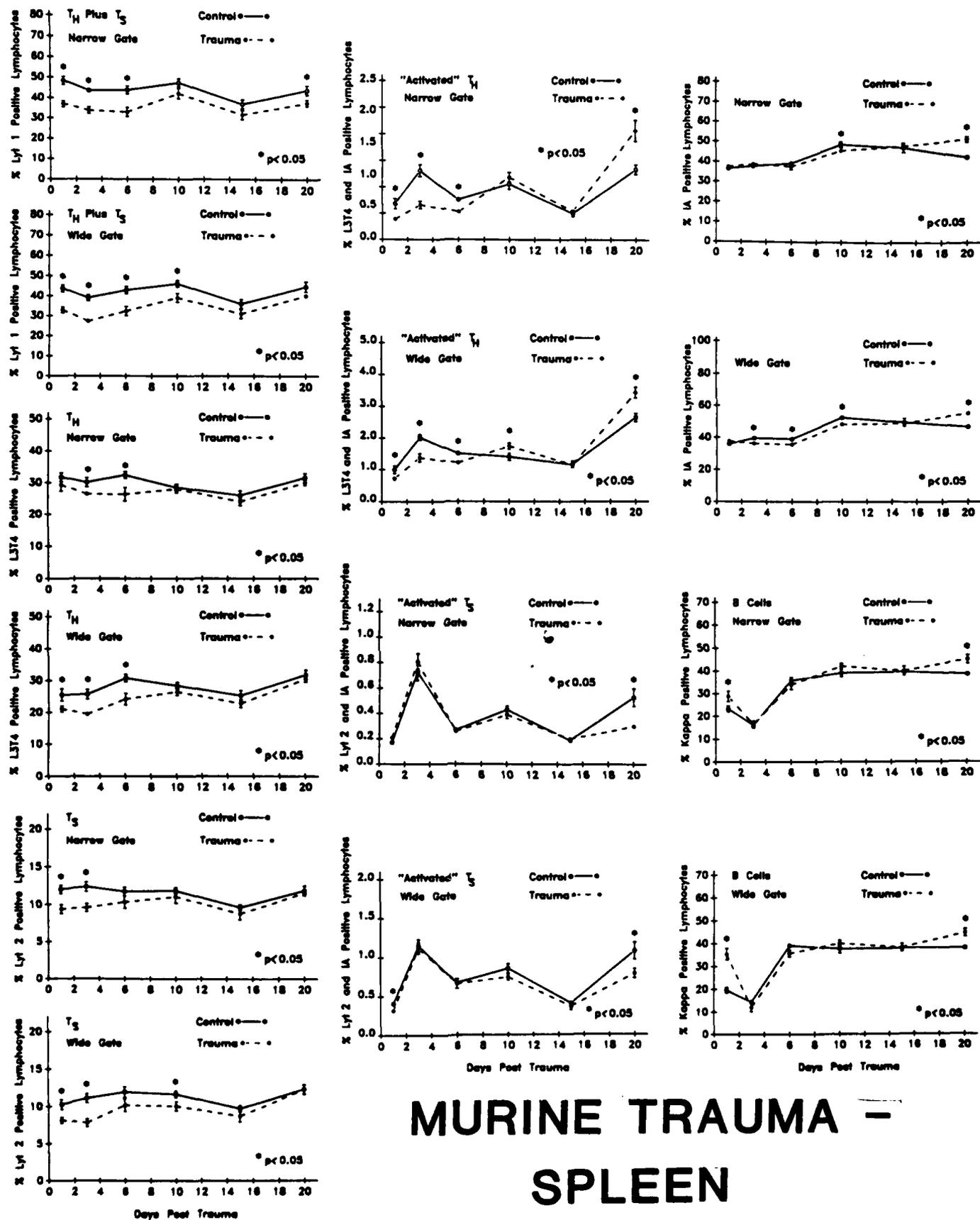
**RESEARCH OBJECTIVES:** 1) Investigation of alterations in immune functions in a murine model of severe trauma (hind-limb crush injury/amputation), to determine which parameters of immune function are altered by this standard model of injury and stress; and 2) Determine if the depressed immune response can be modulated pharmacologically postinjury, particularly by drugs which are known to effect lymphocyte and monocyte activity.

**PROGRESS (Year 02):** In the last year we have completed detailed evaluation of lymphocyte, neutrophil, and humoral immune changes in the murine system following controlled injury. This has involved the setting up and optimizing of several tests of immune functions which are practical and unique. We are now ready to test the effects of various drugs on immune functions in the injured mouse.

**LYMPHOCYTE SURFACE ANTIGEN EXPRESSION IN VIVO:** These studies required much work in the first year and one-half of the project in establishing the immunologic assays. For example, the lymphocyte studies required optimization of labeling techniques with monoclonal antibodies (direct, indirect and combined techniques) and subsequent evaluation by flow cytometry, since we were using small animals and very small amounts of tissue were available. We have now developed techniques which permit two-color, four-parameter flow cytometric analysis on small (500 ul) samples of murine blood and spleen tissue. We have studied in detail expression of the following lymphocyte surface antigens by lymph node, spleen and peripheral blood cells: L3T4 (helper/inducer cells; Lyt-2 (suppressor/cytotoxic cells; Ia (activation and recognition antigen; Il-2 receptor, activation and proliferation marker). These techniques now permit routine identification of all double-combinations of these markers in blood, spleen and lymph node tissue from mice. This detailed analysis is important since lymphocyte activation has been shown to result in expression of novel combinations of antigens by activating and proliferating lymphocytes (References 1-5), and these techniques permit detection of potential activated lymphocyte populations in vivo after injury or other stress. This initial work in establishing these assays is in press in the Journal of Surgical Research.

We then proceeded to study lymphocyte alterations after defined injury in the mouse. Representative data is included, which demonstrates spleen leukocyte changes following musculoskeletal trauma (Figure 1). We have noted a reproducible depression in both helper/inducer (L3T4) and suppressor/cytotoxic (Lyt-2<sup>+</sup>) cells in the spleen after injury. We have also noted a decrease in activated (Ia<sup>+</sup>) helper cells after injury. These changes are very reproducible and may well reflect altered immune regulation after injury. However, they do not indicate which is more important after injury: dysfunction of helper cells or activation of suppressor cells. Further information will be furnished by our lymphocyte blastogenesis studies, as described in the following section.

**LYMPHOCYTE SURFACE ANTIGEN EXPRESSION IN VITRO AFTER MITOGEN STIMULATION:** This work followed our in vivo studies of surface antigen expression in the murine



# MURINE TRAUMA - SPLEEN

FIGURE 1

injury models. It became apparent that it would be necessary to stimulate cells in vitro in order to learn more about their potential to differentiate into immunoreactive cells. This work is based on work in a number of laboratories during the last several years which has shown that defined surface antigens are expressed on lymphocytes followed activation by mitogens or in MLR cultures (Refs. 1-5). Various activation markers (Ia, Il2 receptor) are expressed, and in addition a population of cells emerges which express both helper/inducer (L3T4 in murine, T4 in human) molecules and suppressor/cytotoxic markers (Lyt-2<sup>+</sup> in murine, T8 in human). It is quite interesting that these membrane surface markers are expressed prior to the synthesis of DNA or cell division in stimulated lymphocytes. Very small numbers of these "activated" cells have been demonstrated in peripheral blood in humans.

We have begun an intense effort to study the expression of surface markers on murine lymphocytes following mitogen stimulation (both PHA and ConA). These experiments have shown that the expression of the surface markers is drastically altered after musculoskeletal injury. Representative experiments are shown in Figure 2. These studies suggest that the lymphocytes are in general defective in their ability to express activation antigens on their surface when stimulated at various times after injury, and that the block is at this level rather than a failure simply to proliferate after stimulation. However, it is interesting to find that activated suppressor cells (Lyt2<sup>+</sup>/Ia<sup>+</sup>) are increased on postinjury day 10. The experiments on postinjury day 13 included "control" animals, which had received short duration anesthesia 13 days previously, and "normal" mice, which received absolutely nothing. It appears that even a short period of anesthesia is sufficient to produce profound alterations in surface marker expression 13 days later, compared to completely normal animals. This indicates that non-specific stress has an important immune suppressing action, and we will follow up this observation very soon with further experiments on animals which are anesthetized only (not injured).

Similar experiments are in progress to study expression of the same surface antigens in a one-way MLR, using heat-killed lymphocytes for the stimulator cells and cells from injured mice as the responders.

**SPECIFIC ANTIBODY RESPONSES:** We have studied in detail the specific antibody response to sheep red blood cells in the murine injury models, using a modification of the Jerne plaque assay. Our evidence indicates that the specific response to sheep red blood cells, a T-cell dependent antigen, is moderately elevated after musculoskeletal trauma; serum levels of specific antibody are normal. We have also studied IgG clearance in the mouse after injury and have found shortened half-lives of IgG following injury. We are continuing to correlate these results with our investigations into cell-mediated immunity; we are currently hypothesizing that humoral immunity may be stimulated posttrauma (supported by a number of clinical studies, including our own as noted below) to the detriment of cell-mediated functions. Some of these results will be reported at the Association of Academic Surgery in November 1987, to be published in the Journal of Surgical Research.

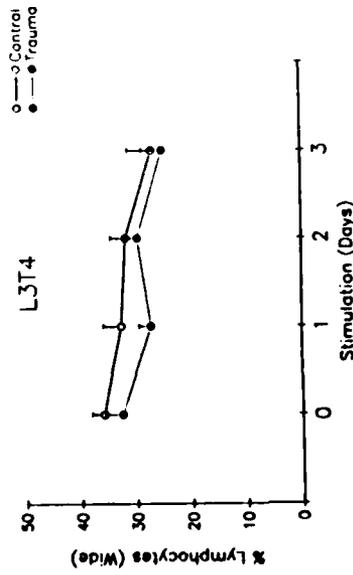
We have now established an assay to study the humoral immune response to LPS (lipopolysaccharide), a T-cell independent antigen. This is done by immunizing animals with LPS and measuring the immune response (plaque-forming cells) by coupling LPS to sheep red blood cells. These experiments are currently in progress. This work is important since regulation of the T-independent response may be affected quite differently by trauma and stress than the T-dependent response.

**OPSONIZATION AND NEUTROPHIL PHAGOCYTOSIS:** We have developed a precise assay using fluorochrome-labeled microorganisms and flow cytometry to measure ingestion of specific microorganisms by neutrophils or monocytes. This assay allows the precise quantitation of ingestion including the number of phagocytic neutrophils and the number of organisms ingested per neutrophil, by analysis of the fluorescent histogram (Figure 3). The unique part of this assay is that any microorganism can

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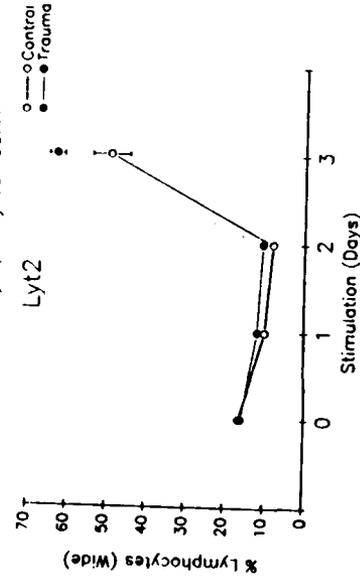
**Injury Day 5**

Murine Splenic Lymphocytes-ConA



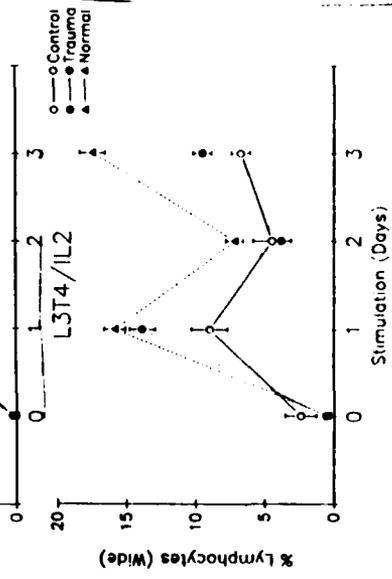
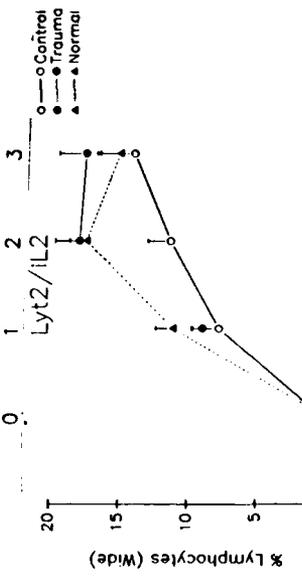
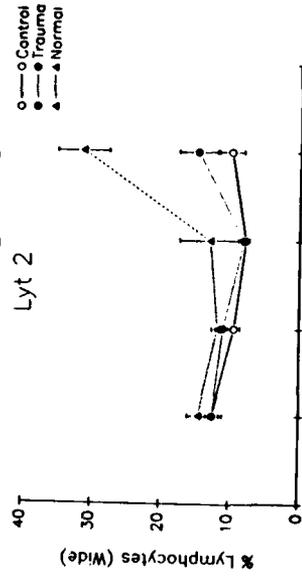
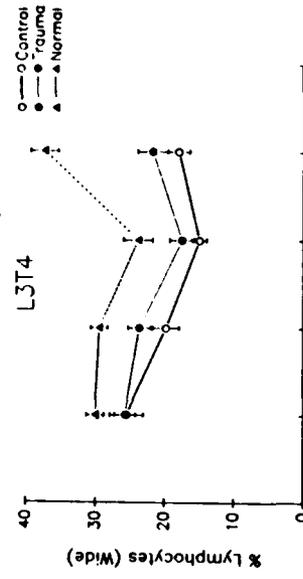
**Injury Day 10**

Murine Splenic Lymphocytes-ConA

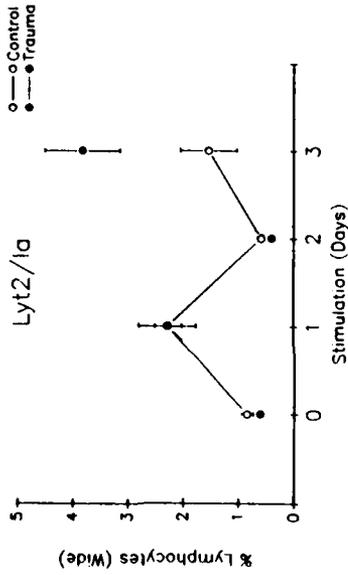


**Injury Day 13**

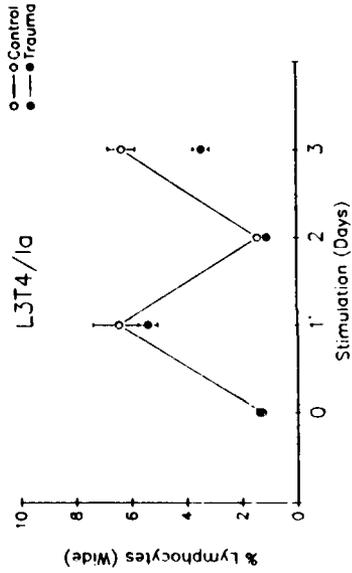
Murine Splenic Lymphocytes-ConA



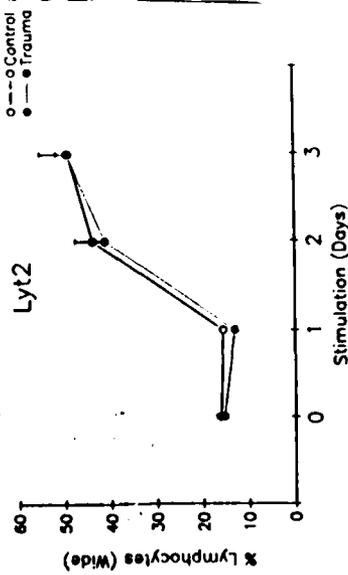
Lyt2/1a



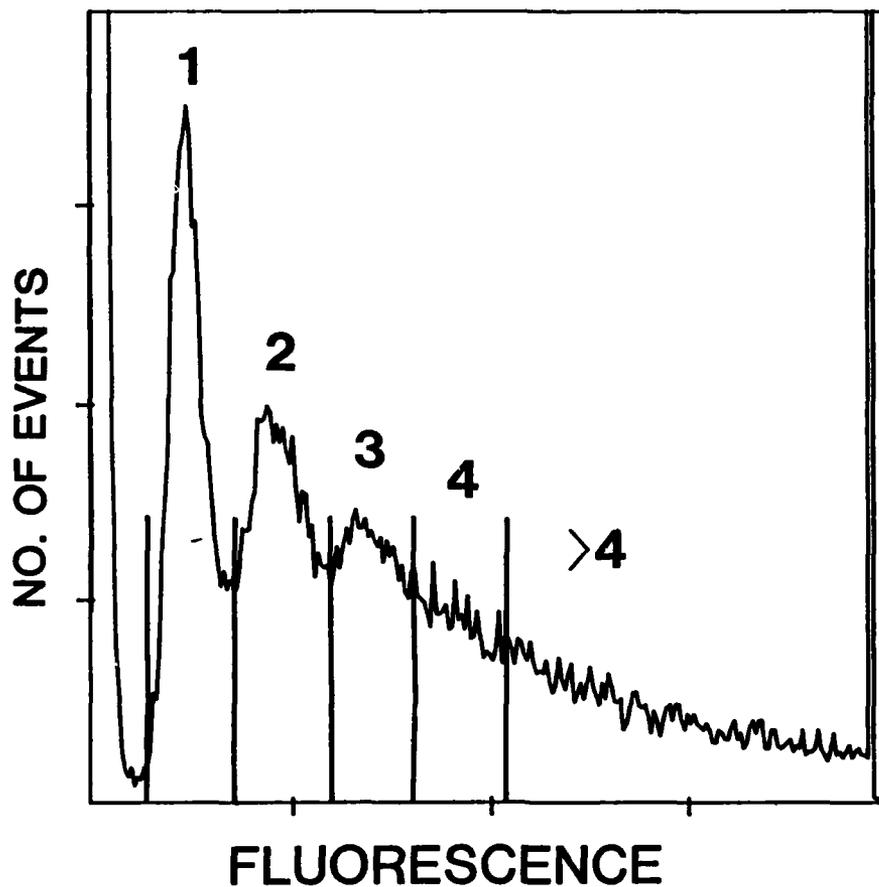
L3T4/1a



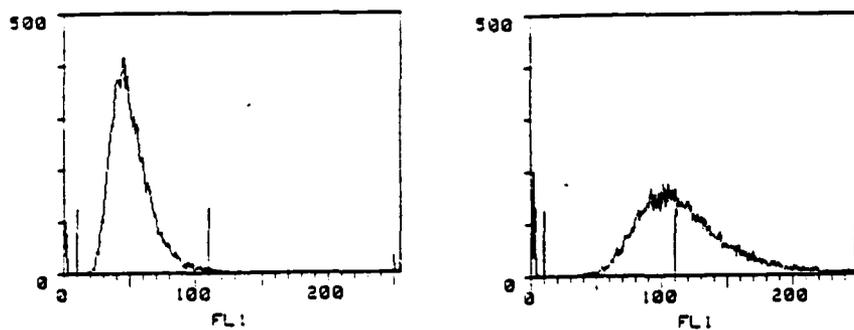
Lyt2



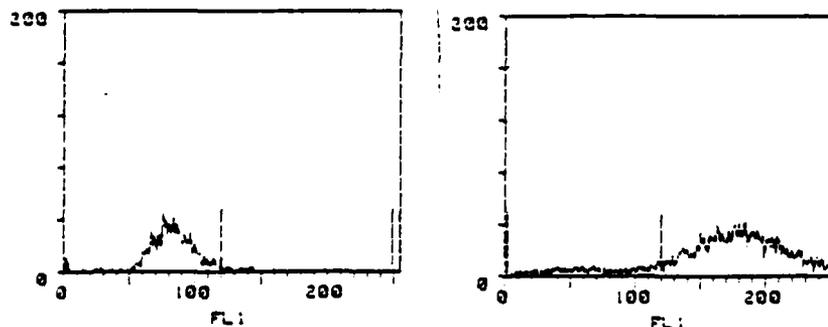
**FIGURE 2**



**FIGURE 3.** Fluorescence histogram of FITC-labeled *Pseudomonas aeruginosa* ingested by murine peritoneal neutrophils. The numbers indicate specific numbers of organisms ingested in that group of PMNs.



**FIGURE 4a.** Chemiluminescence of human peripheral blood neutrophils. Left, no stimulation; right, 15 minutes after stimulation with PMA. 51% of the cells stained green after stimulation.



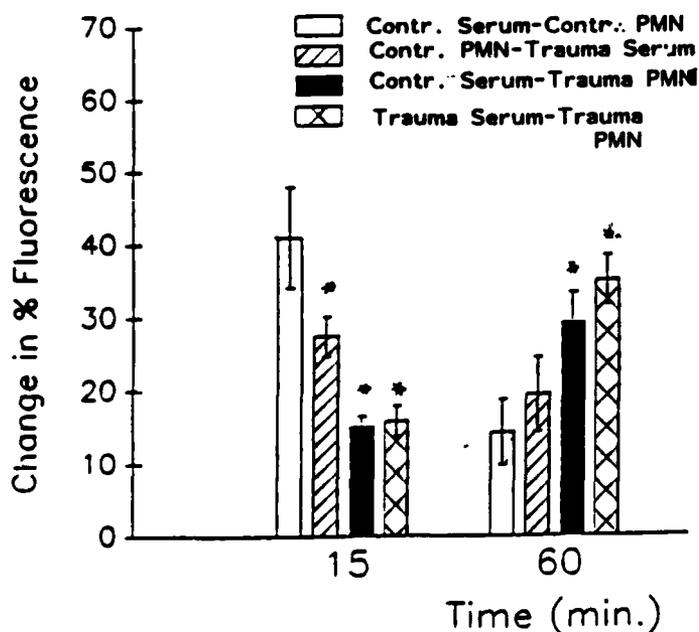
**FIGURE 4b.** Chemiluminescence of murine peritoneal cavity neutrophils. 86% of the cells demonstrate fluorescence with PMA stimulation.

be labeled with the fluorochrome markers and used for the target particle, and we have found that after injury in humans there is defective opsonization of specific organisms but not of all organisms. Detailed description of this assay has been submitted to the Journal of Infectious Diseases. We are currently using this assay to determine osponic and phagocytic defects in the injured mouse, using peritoneal neutrophils.

**NEUTROPHIL CHEMILUMINESCENCE:** We have developed this assay in the last several months to measure  $H_2O_2$  production by neutrophils following particle ingestion or following stimulation with PMA (phorbol myristate acetate). High-energy radicals appear to be one of the primary mechanisms enabling PMNs to destroy engulfed organisms. Since PMNs have been shown to be defective in microbicidal ability after injury, this should be an excellent means to demonstrate such defects and to investigate more fully the reasons for PMN dysfunction. The assay utilizes a dye, 2'7'-dichlorofluorescein, which is taken up by PMNs; when high-energy radicals are subsequently generated in response to PMA, the fluorochrome is altered by  $H_2O_2$ , and light emission occurs in response to laser stimulation. Light emission is readily detected and quantitated by the flow cytometer (6). We have modified the assay for use in the murine system. Figure 4 demonstrates shifts in the fluorescence histogram when chemiluminescence occurs following PMA stimulation, in both murine (peritoneal) and human (circulating) PMNs, thus verifying applicability of the assay to both animal and clinical projects.

We have now developed this assay to the point where it can be used routinely and have demonstrated that trauma in patients and mice results in defective generation high-energy oxygen radicals. This work was submitted in Abstract form in September 1987 to the Society of University Surgeons for publication in the journal, Surgery, pending acceptance. Figure 5 demonstrates striking results in the murine trauma model, indicating that PMNs are markedly defective in chemiluminescence ability and also that factors in serum of traumatized mice can inhibit normal PMN chemiluminescence.

#### MURINE NEUTROPHIL CHEMILUMINESCENCE



**FIGURE 5.** Chemiluminescence in murine PMNs, measured by flow cytometry and the dye 2'7'dichlorofluorescein diacetate after PMA stimulation. Values at 15 min. are much decreased with trauma PMNs and trauma serum. Although the % cells in increased at 60 min, the fluorescence intensity is very weak in the trauma PMNs and trauma serum-PMNs, indicating that those cells are able to activate late but at a very low level of  $H_2O_2$  production.

**WORK PLAN (Year 03):** We will continue to investigate surface antigen expression postinjury using the mitogen-induced blastogenesis assay, using various combinations of cell populations (monocytes and lymphocytes) to determine if certain cell populations are suppressive. We will also develop a similar one-way MLR assay to complement the blastogenesis assay. We will continue to investigate the specific antibody response following injury, using both T-dependent and T-independent antigens, and PMN functions (opsonization, phagocytosis, chemiluminescence). We will test various drugs in injured mice to determine whether these specific immune parameters can be improved by pharmacologic means; these drugs include histamine antagonists, prostaglandin blockers, and cyclophosphamide.

**INVENTIONS (Year 02):** None

**PUBLICATIONS RELATED COMPLETELY OR PARTIALLY TO THIS CONTRACT (Year 02):**

- a. Hansbrough JF, Field, Gadd MA, Soderberg C: Immune response modulation after injury: T-cells and antibodies. *J Burn Care Rehab*, In Press.
- b. Hansbrough JF, Soderberg C et al.: Analysis of murine lymphocyte subpopulations by dual-color flow cytometry: technical considerations and specificities of monoclonal antibodies directed against surface markers. *J Surgical Res* (In Press).
- c. Hansbrough JF, Soderberg C et al: Analysis of murine splenic lymphocyte subpopulations after burn injury by monoclonal antibodies and flow cytometry. Abstract and Presented, Am Burn Assoc Mtg April 1987, Washington DC.
- d. Miller L, Hansbrough JF, Field T: Immunoglobulin kinetics after high-dose replacement therapy in burn patients. Abstract and Presented, Am Burn Assoc Mtg April 1987, Washington DC.
- e. Hansbrough JF: Modulation of the immune response after injury. Abstract and Presented, Immune Consequences of Thermal and Traumatic Injuries, Snowbird Utah, March 1987.
- f. Hansbrough JF: Clinical experience with intravenous gamma globulin in the treatment of burned patients, and pharmacokinetic data. Presented, International Congress on Immune Globulin Therapy, St Petersburg Florida, May 20-23 1987; Manuscript In Press, *Ped Infectious Diseases*.
- g. Hansbrough JF, Field TO Jr, Soderberg C: Analysis of murine peripheral blood T-cell subsets after burn injury using flow cytometry. Abstract and Presented, Internat Congress on Burns, Geneva, June 1987.
- h. Miller L, Field TO, Hansbrough JF: IgG replacement in burn patients: kinetic studies. Abstract and Presented, Internat Congress on Burns, Geneva, June 1987.
- i. Gadd M, Hansbrough JF: Opsonic defects in burn/trauma serum: correction by IgG. Abstract and Presented, Internat Congress on Burns, Geneva, June 1987.
- j. Hansbrough JF, Gadd MA: Measurement of neutrophil phagocytosis and chemiluminescence after injury using flow cytometry. To be presented, Internat Symposium on Wound Healing, Heidelberg, Germany, December 10-12, 1987.
- k. Hansbrough JF: Immunomodulation after burn and trauma in animal models of injury. To be Presented, 1st Internat Congress on The Immune Consequences of Trauma, Shock and Sepsis. Munich, West Germany, March 3-5, 1988.
- l. Hansbrough JF, Soderberg C, Gadd MA: Analysis of murine lymphocyte subpopulations after injury using monoclonal antibodies and two-color flow cytometry. *J Surg Research*, submitted.
- m. Hansbrough JF: H<sub>2</sub>-antagonists and the Immune Response Following Trauma. In *Histamine and the Immune Response*, Ed R Rocklin, Marcell-Decker Inc, In Prep.
- n. Gadd MA, Ozkan N, Hansbrough JF: Serum defects after trauma and thermal injury. Surgery, submitted.
- o. Gadd MA, Hansbrough JF. T cell surface antigen expression after murine injury. Surgery, submitted.

**TRAINING ACTIVITIES:** A surgical resident (Dr. Michele Gadd) has completed one year of full-time research work in our immunology laboratory (July 1986-July 1987) and has elected to spend an additional full year in our laboratory. She is funded by the Department of Surgery and she is spending approximately half-time on the ONR project. One medical student will spend several months in our lab this fall.

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