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Dynamics of T-lymphocyte subpopulations and T-lymphocyte function following thermal injury

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SUMMARY

The study was designed to evaluate T-lymphocyte function in relation to the numbers of T cells present in patients that had sustained major thermal injury. Also, it was designed to determine the dynamics of total T cells and the T-cell subpopulation that formed 'active' E rosettes in separated cell populations. The primary observations were: (a) within 10 days of injury a significant depression of T-cell numbers in separated cell populations occurred, which was paralleled by a decrease in T-cell function. Thus the immunosuppression that has been observed in burn patients appears to have been related to decreases in the numbers of T cells, rather than to any dysfunction at the level of the individual T cell; (b) although T-cell numbers remained depressed, in some patients mitogen responses returned to very high levels, suggesting the possibility that either a highly responsive T-cell subpopulation was selected or that there was a depletion of suppressor cells; (c) in patients that survived there was a gradual return of T lymphocyte numbers to normal levels; and (d) the decreases in the total T-cell population were accompanied by a depletion of the T cells that formed 'active' (37°C stable) rosettes, and the recovery of normal T-cell numbers was accompanied by a disproportionate increase in 'active' rosette-forming cells. The high numbers of 'active' rosettes during the recruitment of new T cells suggested that this T-cell subpopulation may represent cells recently arrived in the peripheral blood from the precursor pools.

INTRODUCTION

Patients that have sustained major thermal injury have been shown to have depressed immunological functions as determined by *in vivo* parameters. Thus homograft survival was prolonged (Kay, 1957; Alexander & Moncrief, 1966) and responses to skin test antigens were depressed (Rapaport *et al.*, 1968). One possible explanation for the depression may be the extensive lymphocytopenia that is observed following major burns (Sevitt, 1957; Casson *et al.*, 1966). Attempts to evaluate that possibility through *in vitro* lymphocyte function testing have produced mixed results. Mixed lymphocyte culture responses using cells from burn patients have been shown to be depressed (Leguit *et al.*, 1973) and depressed PHA responsiveness has been reported (Sakai *et al.*, 1972), but elevated mitogen (PHA) stimulation responses have been observed by others (Leguit *et al.*, 1973; Daniels *et al.*, 1970, 1971; Mahler & Batchelor, 1971). A part of the explanation for the discrepancies may reside in the fact that in contrast to mononuclear cell suspensions derived from normal individuals, which are known to be comprised exclusively of lymphocytes and monocytes, cell populations isolated by similar methods from burn patients were shown to be contaminated by large numbers of immature cells, most of which were granulocyte precursors (Volenec *et al.*, 1977).

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Suppression of T-lymphocyte function has been described in a variety of conditions, including cancer (Garrioch, Good & Gatti, 1970), autoimmune disease (Fudenberg, 1971) and pregnancy (Kasakura, 1971). Several different mechanisms have been postulated to account for immunosuppression, and obviously it is important to define them and determine the different conditions under which various mechanisms are operative. If T-lymphocyte dysfunction actually occurs during an apparently non-immonological condition such as thermal injury, then it is highly relevant. However, if the suppression associated with burns is merely a reflection of decreased T-cell numbers, then that must be clarified. The present study was designed to quantify T cells, and to compare the relative numbers present with mitogenic responses to PHA in cell populations isolated from burn patients at frequent intervals throughout their hospitalization.

Recently, a subpopulation of T lymphocytes has been described which forms 'active' E rosettes (Wybran & Fudenberg, 1973). These cells have been reported to be depressed in patients with cancer (Wybran, Belohradsky & Fudenberg; 1974) and significant changes in percentages have been reported in patients following renal allografts (Kelly & Sheil, 1977). The functional significance of this subpopulation remains unknown, but certain observations may be relevant. 37°C stable E rosettes were formed by the majority of the T lymphocytes in the human thymus (Borella & Sen, 1975); 'active' rosette numbers are depressed in patients with immunodeficiency disease (Wybran & Fudenberg, 1973) and significant increases in 'active' rosette numbers were observed following intratumour BCG inoculation (Lieberman, Epstein & Fudenberg; 1974) and transfer factor therapy (Fudenberg, Wybran & Robbins, 1975). Those observations taken together suggested the hypothesis that T cells that form 'active' E rosettes represent cells recently arrived in the peripheral blood from precursor pools. Because T-cell depletion has been documented in the peripheral blood of patients following thermal injury (Sevitt, 1957; Casson *et al.*, 1966), that condition could represent an optimal situation for evaluating such a hypothesis, e.g. if the hypothesis is correct, it should be possible to see a significant increase in the proportion of 'active' rosettes during the recovery of normal T-cell numbers following thermal injury.

MATERIALS AND METHODS

Patient population. Twenty-seven patients who had sustained second and third degree burns on at least 20% of their body surface were selected for the study because the nature of the injury required prolonged hospitalization and because significant changes occurred in peripheral blood cell compartments in those patients. Normal healthy adults were included as controls for the separation procedure, the T-cell enumeration and the T-cell function studies.

Cell separation. Peripheral venous blood was drawn into Vacutainer® tubes containing 250 μl of preservative-free heparin. Peripheral blood 'mononuclear' cells were isolated by Ficoll–Hypaque density gradient sedimentation (Böyum, 1968). Considerable contamination with erythrocytes occurred in cell populations from burn patients. Those were lysed selectively with Tris–ammonium chloride. Cells were washed three times with Hanks' balanced salt solution (HBSS) and the viability was determined by trypan blue exclusion. A morphological analysis of the separated cells was performed on cytocentrifuged (Shandon–Southern Industries, Sewickley, Pennsylvania) Wright's-stained preparations.

E-rosette assays. The total T-lymphocyte population was enumerated by a standard E-rosette assay (Jondal, Holm & Wigzell, 1972). Briefly, sheep erythrocytes (SRBC), stored in Alsever's solution at 4°C for no more than 1 week, were washed three times with veronal gelatin buffer (VGB) and resuspended to 0·5% in HBSS. The SRBC were mixed with an equal volume of 'mononuclear' cells (10^6 per ml), incubated at 37°C for 5 min and sedimented at 300 g for 5 min. The sedimented cells were incubated at 4°C for 18 hr, gently resuspended and the percentage of cells forming rosettes that consisted of at least three attached erythrocytes was determined, by counting at least 200 cells.

'Active' E rosettes were determined by a modification of the assay originally described by Wybran & Fudenberg (1973). The E-rosette assay was performed as described above but, instead of incubating the SRBC–mononuclear cell mixture at 4°C, the cells were gently resuspended following sedimentation. To insure that only 37°C stable rosettes were counted, the mixture was incubated at 37°C for 5 min and the percentage of rosettes was determined.

T-lymphocyte function assay. A standard phytohaemagglutinin (PHA) stimulation assay was performed. 'Mononuclear' cells were suspended in Eagle's minimal medium supplemented with 10% foetal bovine serum and antibiotics. $5{\cdot}0 \times 10^4$ cells in 0·2 ml of medium were added to each well of ninety-six-well, flat-bottomed Linbro disposotrays (IS-FB-96-TC, Linbro Chemical Co., Hamden, Connecticut). PHA-P (Grand Island Biological Co., Grand Island, New York), at five different concentrations (1, 2, 5, 10 and 25 μl of stock PHA-P/ml), was added to the wells in 10 μl of HBSS. Six replicates were performed for each mitogen concentration as well as for the cell control. Cells were cultured for 72 hr in 5% CO_2 in a humidified incubator at 37°C, 12·6 μCi of [^{3}H]thymidine was added to each well and the culture was continued for 18 hr.

Samples were harvested with a MASH II (Microbiological Associates, Bethesda, Maryland) automated cell harvester, solubilized and prepared for counting with Aquasol® (New England Nuclear, Boston, Massachusetts). All samples were counted in a Mark III beta scintillation counter (Searle Analytic, Chicago, Illinois).

Autoradiography. The morphology of the cells that incorporated [^{3}H]thymidine was determined by autoradiography. Briefly, cells were harvested following mitogen stimulation and [^{3}H]thymidine incorporation, washed three times with HBSS to remove extracellular radioactivity and cytocentrifuged, onto slides that had been pre-cleaned in absolute ethanol, flamed, dipped in gelatin–chrome alum solution and air-dried. The cells were fixed with absolute methanol, air-dried, coated with NTB-3 emulsion (Eastman Kodak Co., Rochester, New York), exposed for 48 hr and developed by standard procedures. The preparations were stained with Wright's stain and the cells with grains over the nucleus were assessed morphologically.

RESULTS

T-lymphocyte dynamics

T lymphocytes were enumerated by E-rosette formation and the accuracy of those determinations was confirmed by counting the number of small lymphocytes in cytocentrifuged preparations. Almost invariably the values for E rosettes and for total small lymphocytes corresponded very closely.

When it was possible, burn patients were tested twice weekly during hospitalization, and the first sample was obtained 3–5 days post-burn. The results of E-rosette testing on several representative patients are presented in Figs 1, 2 and 3. Generally, if cells were obtained and tested within 96 hr of injury, the T-cell numbers were near normal levels. In our laboratory phagocytes were not excluded when E-rosettes were quantified. The mean E-rosette value for 100 normal individuals was $56{\cdot}3 \pm 6{\cdot}8$. Within 4–6 days a substantial decrease in T cells was observed with all patients, although the magnitude of the drop varied from patient to patient. Most patients that were included in the present study exhibited a decrease to the point where fewer than 10% of the isolated cells could be defined as T lymphocytes. Complete blood counts and differentials were performed in parallel and a lymphocyte depletion was observed in most patients.

Patients who failed to recover from their thermal injury generally demonstrated no sustained recovery in the numbers of T cells isolated (Fig. 1 b,d). Patients that demonstrated clinical improvement and ultimately survived their thermal injury exhibited a gradual increase in T-cell numbers. Generally, however, normal levels still had not been reached by the time the patients had recovered sufficiently to leave the hospital.

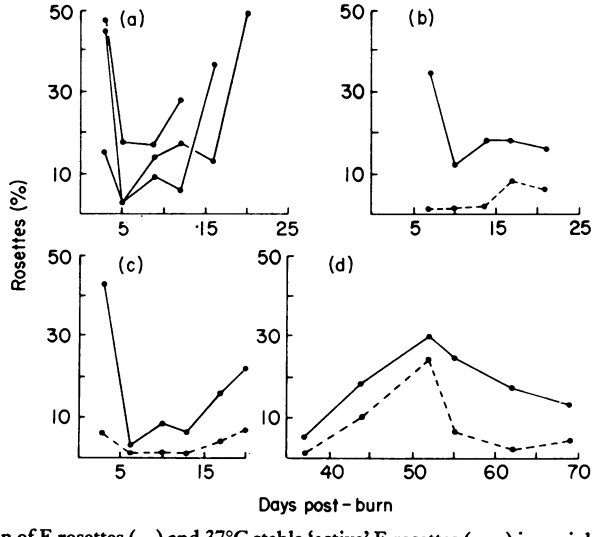

FIG. 1. Quantification of E rosettes (—) and 37°C stable 'active' E rosettes (- - -) in peripheral blood of patients that had sustained major thermal injury. (a) E rosettes were quantified in the peripheral blood of three patients that survived; (b) and (d) both E rosettes and 'active' E rosettes were quantified in peripheral blood of two patients that died; (c) E and 'active' E rosettes in peripheral blood of one patient that survived.

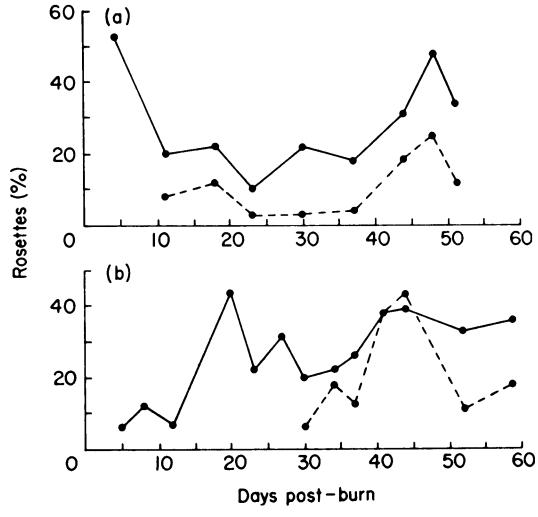

FIG. 2. Quantification of E rosettes (—) and 37°C stable 'active' E rosettes (- - -) in the peripheral blood of two patients that survived major thermal injury.

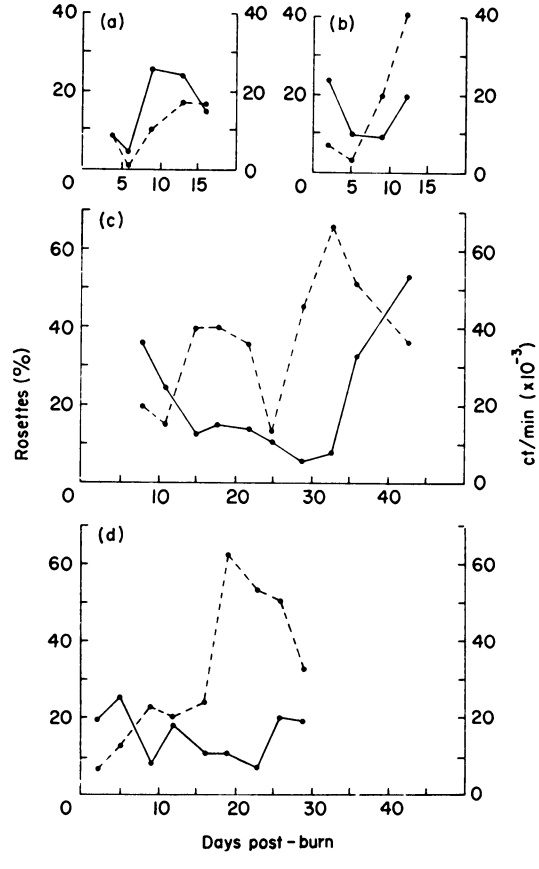

FIG. 3. Quantification of E rosettes (—) and mitogenic response to PHA (- - -) in four patients (a to d) that survived major thermal injury. The mitogenic response, presented in counts per minute (ct/min), has had the unstimulated cell control values subtracted.

T lymphocyte subpopulations–'active' T-cell rosettes

Because nearly total depletion of T lymphocytes was noted in the separated cell population following major thermal injury, burn patients offered an excellent opportunity for observing the dynamics of that T-cell subpopulation as new T cells were recruited. Because the methodology for quantifying 'active' E rosettes has been developed relatively recently, it was important to compare the results obtained with cells from normal individuals in the present study to those obtained in previous studies. In twenty-five tests on normal healthy volunteers, the average percentage of E rosettes was 54 (range: 43–62%) while the average of 'active' E rosettes was 9 (range: 1–20%). The average percentage of the total E-rosetting cell population (T lymphocyte) that formed 'active' rosettes was 17·0.

'Active' rosette levels were followed in several burn patients (Figs 1b,c,d and 2a,b). During the period of significant T-cell depletion the numbers of 'active' rosette-forming cells were very low also. Often, none could be detected (Figs 1d and 2b). However, in patients who recovered and exhibited significant T-cell increases, the numbers of 'active' rosetting T cells increased dramatically, often representing 50–100% of the total rosette-positive population, and the increase generally was coincident with increase in total T lymphocytes.

T-lymphocyte function

For the controls, normal cells always were tested concurrently to verify that the test was working on a technical level. The mean level of stimulation for normal controls (twenty-three) was $31{\cdot}7 \pm 14{\cdot}0 \times 10^3$ ct/min (range: $11{\cdot}3–73{\cdot}5 \times 10^3$ ct/min). An unstimulated control was always included and the counts recorded in Fig. 3 all represent net counts. The cell control has been subtracted. Although five mitogen concentrations were used, only data from one of those concentrations has been presented (5 μl stock PHAP per ml). There was little difference in the degree of stimulation between concentrations except that at the highest PHA-P concentration there was usually some suppression of counts.

The purpose of the present study was to evaluate T-cell function in relation to the number of T cells present. Data from four patients whose T-cell function was evaluated over the duration of their hospitalization were selected as representative and are presented in Fig. 3. Within a few days following thermal injury the lymphocyte stimulation values were significantly depressed, relative both to values obtained with cells from normal individuals that were stimulated at the same time and to values obtained from the same patient at other times of testing. However, in each case, the number of T lymphocytes also was depressed. In a few cases stimulations were observed where the net counts were less than 2000 ct/min. In each of those cases fewer than 5% of the cells could be accounted for as T lymphocytes. In some cases the stimulation values paralleled quite closely the E-rosette values (Fig. 3a and b). However, an unusual phenomenon was observed with several patients (Fig. 3c and d). Despite the presence of very low numbers of T cells, confirmed both by morphology and E rosetting, extremely high counts were obtained. Those high values were not sporadic. Rather, they seemed to follow a pattern, e.g. after a period of depression both in function and numbers there was a substantial increase in function, which was not paralleled by an increase in T-cell numbers.

That was an extremely perplexing finding, and the possibility was considered that the mitogen might be stimulating other cells than T lymphocytes. That was at least a theoretical possibility because large numbers of granulocyte and monocyte precursors were present in the cell suspensions. To evaluate that possibility mitogen-stimulated cells that had incorporated [^{3}H]thymidine were cytocentrifuged and subjected to autoradiographic analysis. Several patients were followed in that manner and in every case, regardless of the time post-burn, the cells that had incorporated [^{3}H]thymidine morphologically resembled lymphoblasts.

DISCUSSION

The results of the present study confirmed that profound changes occur in the T-lymphocyte population following severe thermal injury. T cells were depressed following the injury, mostly as a result of a shift in the types of cells that were obtained by the Ficoll–Hypaque separation method. Depressed T-cell

function appeared simply to reflect that reduction in T-cell number. Confusion with regard to this depression may have arisen from an assumption that cells derived from peripheral blood by Ficoll–Hypaque sedimentation were primarily lymphocytes when, in fact, those cell populations had been contaminated with large numbers of non-lymphocytes (non-mononuclear cells). When patients recovered from the injury there was a recovery of T-cell numbers as well as T-cell function.

The finding of [^{3}H]thymidine incorporation values that were disproportionately high relative to the actual T-cell numbers represented an inconsistency that is difficult to explain. Apparently, the high values did not result from the multiplication of non-lymphocytes. One possible explanation for the results may be that during that time period there was a depletion of a population of regulatory cells. That is, cells which normally function as non-specific suppressors of T-lymphocyte activity were lost. A second possibility may be that T cells vary in their ability to respond to mitogens, and that the subpopulation of T cells that was present was highly responsive to mitogens.

At the beginning of this study it was theorized that the major changes in T-lymphocyte dynamics that occur following major thermal injury would offer an opportunity to evaluate the significance of the 'active' rosette-forming T-lymphocyte subpopulation. The results clearly support, but do not prove, that 'active' rosette-forming T lymphocytes represent cells recently arrived in the peripheral blood from the precursor pool. Thus, following T-cell depletion when T-cell numbers were being reconstituted, presumably through recruitment from the thymus, the proportion of those cells which formed 'active' rosettes was significantly higher than in normal peripheral blood. Generally, they represented over 50% of the total number of T cells. It is possible, therefore, that determination of the percentage of 'active' rosettes in a variety of conditions could represent a measure of T-lymphocyte turnover. Some explanation must be made for the fact that the values obtained for 'active' rosettes in peripheral blood from normal individuals were lower in this study than in previous studies. The normal levels in this laboratory for 'active' rosettes averaged less than 10% while other investigators have obtained average values of 20–30%. The reason was purely technical. It was determined that because T cells from the thymus form rosettes that are stable at 37°C, only 37°C stable active rosettes would be quantified. Thus a 5 min incubation at 37°C, which reduced the number of 'active' rosettes in normal individuals, was added. By decreasing the sensitivity of the assay in that manner, the significance of the very high values for 'active' rosettes in burn patients was increased.

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