Postburn Impaired Cell-mediated Immunity May Not Be Due to Lazy Lymphocytes But to Overwork

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After major trauma, including burns, patients develop a multitude of immunologic alterations, including impaired cellular immunity (CMI). Because of conflicting reports on the relationship of *in vitro* lymphocyte activity to the clinical course of burn patients, we studied CMI in 29 patients with a mean burn size of 41% and a mean age of 32 years. The patients' cellular response to the mitogen phytohemagglutinin and the ability of the patients' serum to suppress a normal lymphocyte mitogenic response were measured. The endogenous level of lymphocyte activity spontaneous blastogenic transformation (SBT) was measured immediately after the cells were harvested from the blood. During the first 72 hours postburn, the ability of the patients' cells to respond to mitogens *in vitro* decreased, while the endogenous activity (SBT) increased. Subsequent changes in the SBT, but not the mitogen-stimulated response, predicted sepsis. Although the patients' serum was mildly suppressive, these changes were not of statistical or clinical significance. It is postulated that the *in vivo* and *in vitro* CMI defects are not primarily due to a defect in the ability of the cell to be activated, but instead are due to exhaustion, desensitization, or down-regulation of these *in vivo*-activated cells.

AFTER MAJOR TRAUMA, including burns, patients develop many alterations in host defense systems, including abnormalities of cell-mediated immunity, humoral immunity, and the nonspecific host defense system.[1] Abnormal cell-mediated immunity has been demonstrated by skin test anergy, prolonged survival of xenografts or allografts, or by failure of lymphocyte mitogenesis. Controversy exists over both the pathophysiology and clinical relevance of these *in vivo* and *in vitro* lymphocyte defects in the burned patient.[2-4] Suggested causes of lymphocyte dysfunction include decreased numbers of helper T-cells, increased numbers of suppressor T-cells, or the presence of circulating humoral suppressive factors,[5,6] including elevated levels of endotoxin and prostaglandin E.[7,8] We hypothesized that these abnormalities were not related to the fact that antigenic challenge at the burn wound preempted lymphocyte activity to such an extent that they became unable to respond to other challenges. It was possible to test this hypothesis by comparing spontaneous blastogenic transformation (SBT) to blastogenic transformation produced by the mitogen phytohemagglutinin (PHA).

Both these test systems measure the incorporation of tritiated thymidine into deoxyribonucleic acid (DNA), and are generally accepted as reflecting lymphocyte dedifferentiation and/or mitotic activity. The SBT measures activity taking place in a group of lymphocytes without exogenous stimulation and, thus, measures endogenous or "natural" stimulation. The SBT has been used extensively for this purpose in transplantation studies.[7] PHA-stimulated blastogenosis is frequently expressed as the stimulation index which is a ratio of PHA stimulation/SBT, and reflects the additional capacity of lymphocytes to respond above the "naturally" occurring activity. Thus, either a high denominator or a low numerator will result in a low stimulation index. By measuring both the unstimulated SBT and stimulated PHA blastogenesis, we could correlate the level of the patients' endogenous lymphocyte activity with the ability of the cells to be stimulated *in vitro,* and evaluate the entire equation.

In addition to measuring these cellular responses, the effect of the patients' serum on control lymphocyte activity was measured to determine if the serum had suppressive activity, and to correlate any serum suppressive activity with the cellular responses. The results of our studies documented that the patients' SBT was increased while the mitogen-stimulated activity was normal or decreased. The level of endogenously stimulated lymphocyte activity (SBT) accurately reflected the patients' clinical status, including the onset of sepsis. These results indicate that the *in vitro* lymphocyte defects are not exclusively due to a nonresponsive "lazy" cell, but

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due to *in vivo* exhaustion, desensitization, or down-regulation of lymphocytes.

Patient Population

A total of 29 patients, 20 male and 9 female, were studied. The mean age of these patients was 22.3 ± 13.2 years and the mean total body surface area (TBSA) burn size was 41.4% ± 20.8, with a third degree component of 16.2% ± 17.8. Ten of the 29 patients (34%) became setpic at some time during their hospital course and five patients died for a mortality rate of 17%. The mean TBSA of the patients who died was 60%, with a mean third degree component of 43%. Three of the five patients who died had pulmonary burns.

All patients were treated with a standard therapeutic regimen, which was modified according to the clinical response of each patient. This protocol consisted of fluid resuscitation based on the Parkland Formula, topical antimicrobial agent administration (sulfadiazene silver or mafenide acetate), and nutritional support based on each patient's estimated caloric requirements. Nutritional support was primarily by the enteral route, although peripheral and central hyperalimentation was used when enteral alimentation alone was not sufficient. Prophylactic antibiotics were not used routinely. All deep partial- and full-thickness burns were excised and grafted as soon as the patients were hemodynamically stable. The initial operation was performed usually between the second and sixth postburn days. Burn wound sepsis was treated with parenteral antibiotics, a change in the topical agent, and surgical excision 12 to 24 hours later.

Materials and Methods

Cell preparations. Fifteen milliliters of heparinized blood were drawn from patients at appropriate intervals. The blood was layered over a ficoll/hypaque gradient and centrifuged ×400 g for 30 minutes. The mononuclear cell layer was removed and adjusted to a concentration of 4×10^6 cells/ml in a 20% solution of human AB serum, in minimal essential medium. Viability was tested by eosin red dye exclusion and was always greater than 95%. Lymphocytes were obtained from putatively normal adult volunteers in the same manner and served as control cells.

Cell cultures. All cultures were established in microtiter plates with 50 μl of cell suspension (2×10^5 cells) per culture well. The cultures were maintained at 37 C in a 5% CO_2 environment. At appropriate times, the cultures were pulsed with 2.5 μCi of tritiated thymidine, incubated a further 18 hours, harvested with a Cook cell harvester, precipitated, washed, and the incorporated radioactivity determined with a scintillation counter and recorded as counts per minute (cpm). All assays were performed in triplicate and the results were averaged.

Serum. Serum was obtained from clotted tubes of whole blood allowed to stand at 20 C for 60 to 90 minutes.

Spontaneous blastogenic transformation (SBT). The lymphocyte suspensions were placed into the cell culture trays and immediately pulsed with tritiated thymidine. The incorporated label was determined after 15 hours in culture. A companion culture was performed simultaneously with lymphocytes obtained from a normal volunteer.[9] The patient's SBT, which measures endogenous simulation, was compared to that of a normal volunteer and expressed as the SBT ratio.

The SBT ratio was computed by dividing the patient's SBT in cpm by the control SBT run the same day.

$$\text{SBT ratio} = \frac{\text{SBT cpm:patient}}{\text{SBT cpm:control}}$$

An SBT decay ratio (SBT-DR) was computed to determine whether the level of endogenous blastogenic transformation was affected by placing the cells in tissue culture.

$$\text{SBT} - \text{DR} = \frac{\text{SBT (pulsed at 0 time)}}{\text{SBT (pulsed after 5 days of culture)}}$$

Mitogen-stimulated blastogenic transformation (MSBT). This assay was a modification of the techniques described by Constantian et al.[6] Cultures were established containing 2×10^5 lymphocytes per well. PHA was added to sequential wells at the following doses: 0, 0.5, 5, 10, and 40 μg per well. After 72 hours, the cultures were pulsed and the incorporated label determined after 18 hours.

The MSBT results were expressed in two ways. The first method of expressing the data compared the patients' mitogenic response (MR) against that of control cells studied in parallel. This MR compared the absolute mitogenic activity of the patients' cells in cpm against the control cells, and is similar to the SBT ratio.

$$\text{Mitogen ratio} = \frac{\text{cpm (patients' cells with PHA} - \text{patients' cells without PHA)}}{\text{cpm (normal cells with PHA} - \text{normal cells without PHA)}}$$

The stimulation index (SI) reflects the ability of the cells to be stimulated by PHA. In this calculation, the maximum cpm recorded with any of the PHA doses was divided by the cpm of the same cell unstimulated (O PHA), for example:

TABLE 1. *Effect of Thermal Injury on Endogenous and Mitogen-stimulated Lymphocyte Blastogenic Activity During the First 72 Hours Postburn*

		Endogenous Activity		*In Vitro*-stimulated Activity		
	N	SBT (cpm)	SBT Ratio	MSBT (cpm)	Mitogen Ratio	SI
Control	15	346 ± 48	1	31,571 ± 4235	1	100 ± 17.4
Patient	17	677 ± 109*	1.9 ± 0.3*	20,371 ± 3885†	0.8 ± 0.1†	52 ± 8.9†

All values expressed as Mean ± SEM. SBT = spontaneous blastogenic transformation; cpm = counts per minute; MSBT = mitogen-stimulated blastogenic transformation; MR = mitogen ratio; and SI = stimulation index.

* $p < 0.01$ *versus* control.

† $p < 0.05$ *versus* contol.

$$SI = \frac{\text{maximum cpm with PHA}}{\text{cpm without PHA}}$$

The higher the SI, the greater the magnitude of stimulation above background.

If the endogenous level of lymphocyte activity (SBT) decayed while the cells were in tissue culture, the SI would overestimate the mitogenic reserve of uncommitted cells. Therefore, a modified SI (MSI) was computed which used the SBT values instead of the 5-day O PHA cuture values as the denominator in the SI equation.

$$MSI = \frac{\text{maximum cpm with PHA}}{\text{SBT cpm}}$$

To determine the effect of the patients' serum on control lymphocyte blastogenesis, the patients' serum was added to control cell cultures to yield a 10% v/v concentration in the experimental assay. The control assay was supplemented with pooled normal serum (10% v/v). The lymphocyte blastogenic assay was then performed. The per cent of serum suppression was determined from the following formula.

$$\text{Per cent Serum Suppression} = 100 \times 1 - \frac{\text{cpm ([patient serum with PHA] − [patient serum without PHA])}}{\text{cpm ([normal serum with PHA] − [normal serum without PHA])}}$$

Statistical Analysis

Results were expressed as the Mean ± SEM unless otherwise specified. The unpaired Student's t-test or chi-square analysis were used to determine if the results for various groups were statistically different. Linear regression analysis was performed to characterize the statistical relationship between the severity of the thermal injury and the magnitude of the initial SBT and mitogen response. Statistical significance was achieved at the $p < 0.05$ level.

Results

Cellular Activity

The data have been analyzed and grouped into one of three categories to reflect the temporal and clinical patterns of stress which occur after a major thermal injury. These three categories include the acute injury phase (first 72 hours postburn), the nonseptic hospital period, and periods of sepsis. During the first 72 hours postburn, there was an increase ($p < 0.01$) in the endogenous activity of the patients' cells measured as SBT (Table 1). Concomitant with this increase in the SBT was a significant decrease in the stimulated mitogenic response of the patients' cells to PHA ($p < 0.05$) (Table 1). The initial decrease in stimulated activity did not correlate significantly with the severity of the injury, either when the patient's predicted mortality was compared with the SI ($p = 0.16$) or with the MR ($p = 0.18$). Similarly, neither the predicted patients' mortality ($p = 0.10$) nor the size of the burn ($p = 0.20$) correlated with the initial values of the SBT.

The initially elevated SBT ratios rose significantly during the hospital course of these patients ($p < 0.01$), especially during septic episodes (Fig. 1). Although the SI and the MR were lower in the patients during septic episodes than when the patients were not septic, these differences were not statistically significant ($p = 0.30$) (Table 2, Fig. 2).

Since the SI is the ratio of mitogen-stimulated activity divided by endogenous activity, either a decrease in the numerator or an increase in the denominator would result in a low SI. Therefore, we performed experiments to determine whether the endogenous activity of the patients or the control cells would change after 4 days in tissue culture. These results are shown in Table 3. The control cells demonstrated a minimal change in spontaneous activity after being cultured for 4 days, while the spontaneous activity of the patients' cells was 9.2 ± 1.6 times higher immediately after being harvested than after 4 days in culture. The spontaneous endogenous cellular activity of the control cells decreased an average

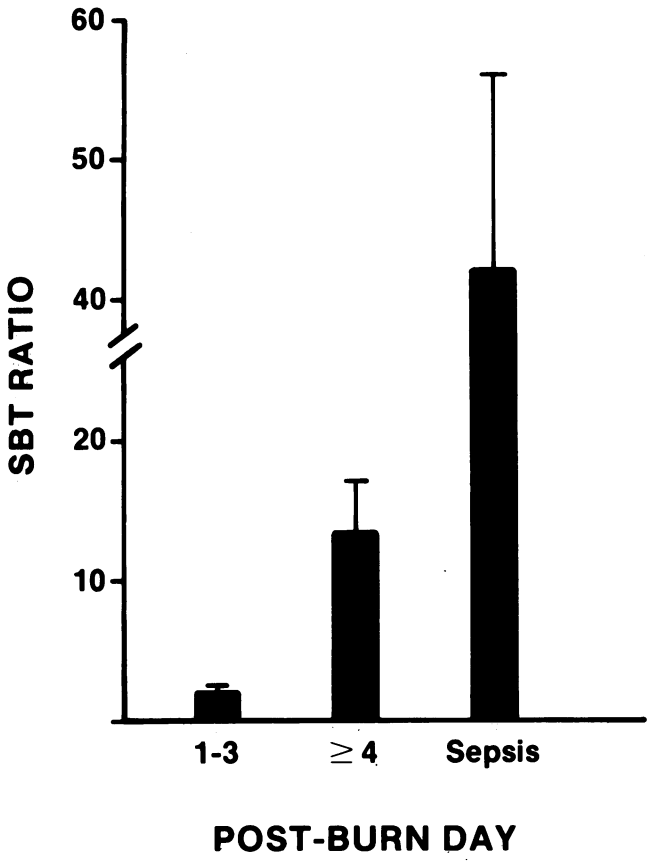

FIG. 1. Spontaneous blastogenic transformation ratio of burn patients stratified according to the time postburn cells were tested. All values expressed as Mean ± SEM.

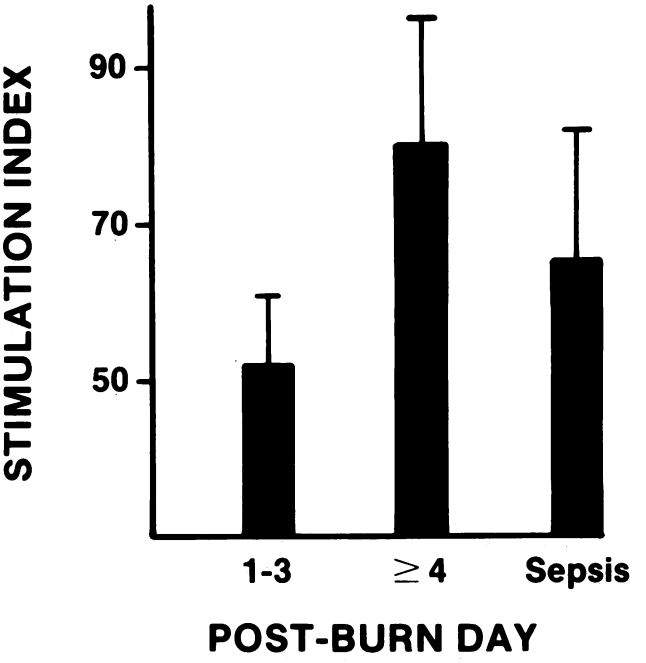

FIG. 2. Stimulation index of burn patients stratified according to the time postburn cells were tested. All values expressed as Mean ± SEM.

of 25% during the period the cells were in tissue culture, compared with an average decrease of 89% of the patients' cells. In spite of the fact that the patients' resting cell activity decreased more than the control cells after 4 days in culture, it remained 270 ± 40% higher than the control cells ($p < 0.05$). These results indicated that the patients' spontaneous blastogenic activity decreased significantly during the period of time the cells were in culture. This was especially true during septic episodes ($p < 0.01$, Table 3).

Since the endogenous activity (SBT) of the patients' cells decreased markedly after 4 days of culture, the traditional method of computing the SI by using the resting activity of the cultured cells might not accurately reflect cellular immunity. Therefore, we computed the SI using the SBT values obtained immediately after the cells were harvested as the resting cell level, rather than the resting levels obtained after 4 days in culture without PHA. This value was called the modified stimulation index (MSI) to distinguish it from the stimulation index (SI). The MSI of the control cells remained high, with a mean value of 104 ± 13, while the patients' cells had MSI of only 19 ± 3.6 ($p < 0.001$). The greatest decrease in the MSI was seen when the patients were septic. In this circumstance, the MSI was 3 ± 1 ($p < 0.01$) (Table 3). These results suggested that although the cells were activated *in vivo* postburn, they could not be stimulated *in vitro* by PHA as well as control cells which have not been activated *in vivo*. The cells obtained from patients during septic episodes were especially resistant to further *in vitro* stimulation by PHA.

TABLE 2. *Effect of Sepsis on Endogenous and Mitogen-stimulated Lymphocyte Blastogenic Activity after the First 72 Hours Postburn*

		Endogenous Activity		*In Vitro*-stimulated Activity		
	N	SBT (cpm)	SBT Ratio	MSBT (cpm)	MR	SI
Control		265 ± 38	1	27,521 ± 2,022	1	113 ± 23
Patient	26	3550 ± 860*	13.4 ± 3.7*	26,254 ± 2,108	1.0 ± 0.1	80 ± 16
Patient during sepsis	9	11,250 ± 2900*†	42 ± 16*†	21,527 ± 2,834	0.8 ± 0.2	65 ± 17‡

See Table 1 for abbreviations.
* $p < 0.01$ *versus* control.
† $p < 0.05$ *versus* nonseptic.
‡ $p = 0.07$ *versus* control.

Serum Activity

Seventy-four serum specimens from 24 patients were tested for suppressive activity in the blastogenic assay using control leukocytes. Overall, these serums suppressed control blastogenic activity by 17%. Sixty of the specimens were collected when the patients were not infected, and the mean SD suppressive activity was 15% ± 4.4 *versus* 28% ± 11.2 during 14 septic episodes ($p = 0.10$). Thus, although serum suppression of blastogenesis was greater during sepsis, this difference was not statistically significant. However, if a level of suppression of greater than 40% of control was considered positive, then 54% of the serum samples collected during sepsis were suppressive, in contrast to only 10% of the samples when the patients were not septic ($\chi^2 = 11.2$, $p < 0.01$). Although the patients, as a group, were more likely to have suppressive serum when they were septic, this finding was not helpful in diagnosing sepsis in the individual patient.

Clinical Utility of SBT Ratio as Predictor of Sepsis

Figures 3 and 4 illustrate the association between the SBT ratio, MR, or serum suppressive activity with the clinical course of two patients. The first patient sustained a 75% TBSA burn with 60% full-thickness injury. She became septic on the 15th postburn day and died on day 33 of sepsis. There was no change in her MR when she initially became septic and, in fact, after 10 days of intermittent septic episodes her MR was increased. Preterminally, she demonstrated a profound fall in her MR, and her serum at that time was suppressive to control cells. Neither serum suppressive activity nor mitogen ratio correlated with this patient's clinical course. In contrast, the SBT rose markedly when this patient became septic and remained high until 48 hours prior to her death (Fig. 3). The second patient, a 23-year-old man with a 43% total body surface area burn with 23% full-thickness injuries, had two septic episodes during his hospital course. In this patient, the clinical onset of sepsis was associated with a marked elevation of the SBT ratio, which returned to normal promptly after the septic episodes had resolved. Neither the patient's mitogen ratio nor his serum suppressive activity consistently reflected the clinical course.

In our experience, an elevated SBT ratio was the best indicator of sepsis. When the SBT ratio was greater than 20, sepsis was present in these patients with two exceptions (Fig. 5, $p < 0.001$). In the two patients whose SBT ratio was less than 20 during sepsis, this was a preterminal event. The first patient was described in Figure 3, and the decrease in SBT ratio occurred as a preterminal

TABLE 3. *Relationship Between Endogenous and Mitogen-stimulated Activity after Four Days in Tissue Culture*

	SBT-DR	MSI
Control	1.3 ± 0.3	104 ± 13
Patients	9.2 ± 1.6*	19 ± 3.6*
During sepsis	20.2 ± 4.0†	3 ± 1.0†

All values expressed as Mean ± SEM. SBT-DR = spontaneous blastogenic transformation decay ratio; MSI = modified stimulation.
* $p < 0.001$ *versus* control cells.
† $p < 0.01$ *versus* control cells and patient cells.

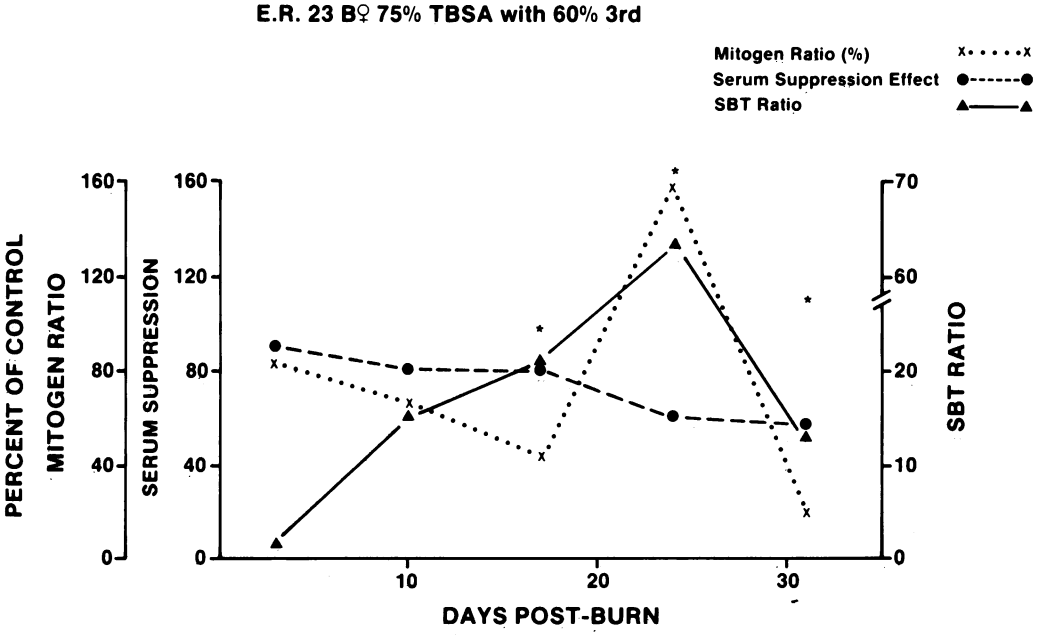

FIG. 3. Clinical profile of a 23-year-old black woman with a 75% burn who died on the 33rd postburn day of sepsis. *Denotes periods of sepsis.

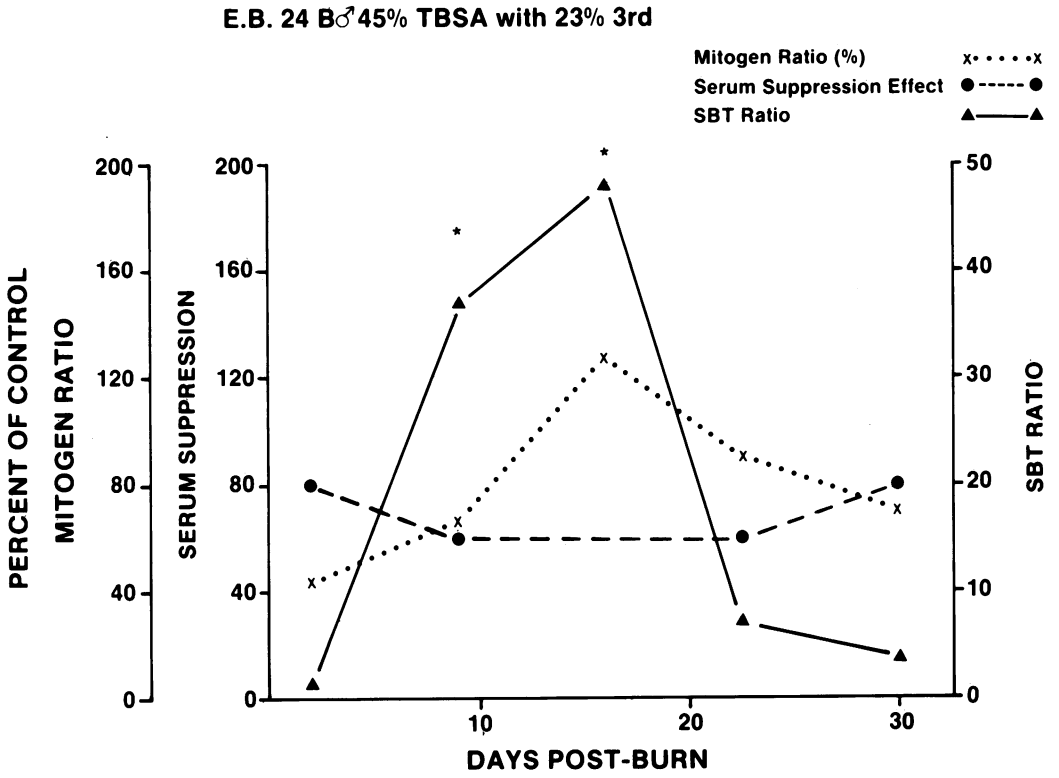

FIG. 4. Clinical profile of a 24-year-old black man with a 45% burn who was discharged on the 36th postburn day. *Denotes periods of sepsis on postburn days 10 and 18.

event. Similarly, the SBT ratio fell from 25 to 5 preterminally in a 53-year-old black woman with a 43% burn. Thus, in both instances, when the SBT was less than 20 during sepsis, this event occurred within 48 hours of the patients' death.

Discussion

A fundamental role of the immune system is the preservation of the body's integrity against invading microorganisms. The burn patient is uniquely at risk for the invasion of microorganisms due to loss of the local skin barrier, combined with widespread defects in systemic host defenses. Beginning with the observation that a burn victim may have delayed rejection of skin allografts, many studies have been performed to evaluate cell-mediated immunity in the burn patient. Although many investigators have documented impaired cell-mediated immunity either as *in vivo* skin test anergy or as *in vitro* failure of SBT, there is no consensus on whether *in vitro* cell-mediated immunity is normal,[2] increased,[3,10] or decreased.[4,11-13] Controversy exists over whether changes in the cellular response to PHA correlate prognostically with sepsis or death.[2,11,14-16] To a large degree, the differences in these studies are related to differences in patient care protocols, patient selection, methodology, and data analysis.

Based on our study of 29 patients, we found that neither the level of the patients' stimulated lymphocyte activity, whether measured as the SI, MR, or CPMs, nor the magnitude of serum suppressive effect of the patients' serum on stimulated control lymphocyte activity reliably identified which patients would become septic or die of their injuries. Our cellular results are consistent with the work of some investigators,[2,11] who could not correlate changes in the cellular response to PHA with sepsis or death, but in contrast to the results of other investigators, who have reported that the magnitude of the depression in the PHA cellular response was less in survivors than in nonsurvivors.[15] However, these latter investigators did not use early excision and grafting in their treatment protocol, but began operating on the patients between the 20th and 25th postburn day. In contrast, we began operatively to close the wound within the first postburn week. Serum specimens collected when our patients were not septic suppressed control lymphocyte activity 15% *versus* 38% during septic episodes. Although serum suppression of blastogenesis was greater during sepsis, this difference did not reach statistical significance ($p = 0.10$). Our results agree with Mannick's group, who reported suppressive serum was common after thermal injury and did not correlate with infection or survival.[4,15] Ninneman et al. found that the incidence of suppressive serum was related to the severity of the injury,[17] and that essentially all patients with burns greater than 50% TBSA third degree have suppressive serum.[7] The difference in treatment protocols

could partly explain these discordant results since, in laboratory models, early excision of the burn wound is associated with the restoration of thymic activity[18] and increased resistance to infection.[19]

A potential weakness of all standardized *in vitro* assays is the risk that the assay may not accurately reflect the complex interactions that are occurring *in vivo*. Thus, in addition to performing the *in vitro* lymphocyte and serum blastogenic assays, we also measured the level of lymphocyte endogenous activity as the SBT. Immediately after the thermal injury, the patients' endogenous SBT was increased compared to control cells, in spite of a decrease in the ability of the patients' cells to be stimulated by PHA *in vitro* (Table 1). The SBT increased during the hospital course and was highest during septic episodes (Fig. 1, Tables 2 and 3). In our series of patients, an increase in the endogenous level of lymphocyte activity (SBT) correlated with sepsis. This hypermetabolic response of the patients' lymphocytes, which is similar to the hypermetabolic response seen in other organ systems after thermal injury, especially during sepsis, could be a nonspecific response or represent the consequence of local antigenic stimulation at the burn wound.

Our results documenting an increased level of cellular activity after thermal injury are consistent with a recent report indicating that lymphocytes from burn patients are spontaneously producing elevated levels of immunoglobulins *in vitro*, which cannot be further augmented by *in vitro* mitogen stimulation.[20] When we examined the effect of placing the cells in tissue culture, it was apparent that, after 4 days in tissue culture, the patients' spontaneous lymphocyte activity had decreased 89% in contrast to 25% in the control cells. Thus, removal of the cells from the *in vivo* burn environment was associated with a major decrease in spontaneous cellular activity. The influence of the burn environment on cellular activity has also been documented by Ninnemann,[21] who reported that when mononuclear cells from burn patients were allowed to rest in tissue culture for 48 hours before PHA stimulation, they would respond better to PHA stimulation than cells immediately stimulated after being harvested from the blood.

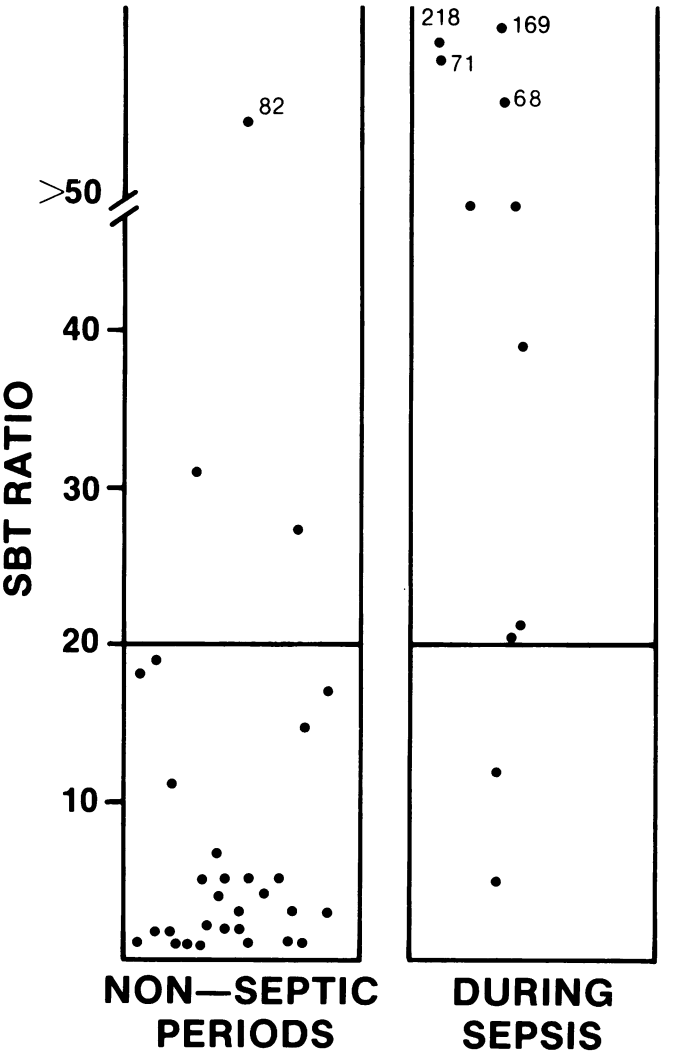

FIG. 5. Scattergram of SBT ratio comparing nonseptic to septic periods. An SBT ratio greater than 20 was associated with sepsis ($p < 0.001$).

Conclusion

Based on our results, it appears that impaired cell-mediated immunity is not due to a defect in the ability of the cells to be activated. Instead, the increased level of *in vivo* lymphocyte activity, as reflected by an increased SBT, indicates that the lymphocytes are being activated *in vivo* and are not sluggish or lazy. Three major hypotheses have been proposed to explain the acquired defect in cell-mediated immunity, which occurs after thermal injury. These hypotheses include the presence of circulating humoral suppressor factors,[6–8] a decrease in the number of helper T-cells,[5] and an increase in the number of suppressor T-cells.[22–24] The fact that the immunosuppressive capacity of the patients' serum does not correlate with the levels of spontaneous or stimulated cellular activity in our patients, as well as in other series,[4,11] casts doubt on the thesis that circulating humoral factors are principally responsible for the observed changes in cell-mediated immunity. The increased level of spontaneous blastogenic activity observed in our patients could have been due to the selective *in vivo* activation of suppressor cells, as proposed by other groups.[4,12,25,26] However, our results can be explained without invoking changes in lymphocyte subsets, since the biological phenomenon known as antigenic compe-

tition[27] could explain the combination of increased endogenous activity associated with a depressed stimulatory response. In antigenic competition, cells that have already been stimulated become relatively resistant to further stimulation. The hypothesis of *in vivo* stimulation leading to an impaired *in vitro* response is consistent with our findings using PHA as a probe, as well as with the work of Shorr et al.,[20] who documented a similar phenomenon in immunoglobulin production after thermal injury.

Cellular exhaustion or down-regulation of helper T-cells could also explain the combination of an increased spontaneous blastogenic activity associated with a normal or decreased SI. This possibility is supported by recent evidence documenting that interleukin-2 (a lymphokine produced by helper T-cells) is decreased after thermal injury, since this lymphokine is important in triggering the proliferation of activated T-cells.[28,29] It is not possible to say with certainty which of these alternatives, if any, is occurring at the cellular level. However, a clear understanding of these immunological changes at the cellular level is critical for the development of rational therapeutic modalities to augment host resistance to infection.

In conclusion, based on the results of our study, we postulate that the *in vivo* and *in vitro* cell-mediated defects, when present, are not primarily due to a failure of lymphocyte activation (the lazy lymphocyte), but may be due to cellular exhaustion, desensitization, or down-regulation of *in vivo*-activated cells. Furthermore, the level of *in vivo* endogenous activity, measured as the SBT, more accurately reflects the clinical state of the patient than does the ability of the cells to be activated *in vitro* by the mitogen PHA.

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