In Vitro Cell-Mediated Immunity after Thermal Injury Is not Impaired

Density Gradient Purification of Mononuclear Cells Is Associated with Spurious (Artifactual) Immunosuppression

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Mononuclear cells isolated by density gradient centrifugation from the peripheral blood of burn patients, but not healthy volunteers, are contaminated with large numbers of nonmononuclear cells. These contaminating leukocytes could cause artifactual alterations in standard *in vitro* tests of lymphocyte function. Thus, we compared the *in vitro* blastogenic response of density gradient purified leukocytes and T-cell purified lymphocytes from 13 burn patients to mitogenic (PHA) and antigenic stimuli. The mitogenic and antigenic response of the patients' density gradient purified leukocytes were impaired compared to healthy volunteers ($p < 0.01$). However, when the contaminating nonlymphocytes were removed, the patients' cells responded normally to both stimuli. Thus, density gradient purified mononuclear cells from burn patients are contaminated by leukocytes that are not phenotypically or functionally lymphocytes. Since the lymphocytes from burn patients respond normally to PHA and alloantigens after the contaminating nonlymphocyte cell population has been removed, it appears that *in vitro* assays of lymphocyte function using density gradient purified leukocytes may give spurious results.

SHORTLY AFTER it was first recognized that allogeneic and xenogenic skin grafts survive for prolonged periods in burn victims,[1] it was postulated that impaired cell-mediated immunity might predispose these patients to septic complications. Over the past decade, using increasingly sophisticated *in vitro* technology, investigators have attempted to define and understand the mechanisms responsible for acquired immune defects documented after thermal injury. Humoral suppressive factors[2,3] increased suppressor T-cell activity,[4–6] decreased helper T-cell activity,[7] altered cytokine production,[8,9] and failure of interleukin-2 (IL-2) production and receptor expression[10] have all been proposed as reasons why cell-mediated immunity is impaired after thermal injury. In contrast, other investigators have not found evidence of impaired cell-mediated immunity *in vitro*.[11] Thus, despite an intensive investigative effort, confusion and controversy continue.

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We have recently reported that mononuclear cells isolated by density gradient centrifugation from the peripheral blood of burn victims, but not healthy volunteers, are contaminated with large numbers of metabolically active cells that are not functionally or phenotypically lymphocytes.[12] Knowledge of whether these activated leukocytes alter the response of mature lymphocytes *in vitro* or modify the immune response *in vivo* may be important. Furthermore, since these activated leukocytes spontaneously take up thymidine but do not respond to phytohemagglutinin (PHA),[12,13] the presence of large numbers of these contaminating leukocytes could potentially cause artifactual alterations in the results of standard *in vitro* tests of lymphocyte function. In fact, since most of the studies on lymphocyte function reported to date use density gradient seperation techniques to obtain their mononuclear cell population, it is unknown whether the results of these *in vitro* tests can be extrapolated to the *in vivo* state. Thus, in order to determine whether the lymphocyte response of the burn patient is truly different from that of healthy controls, it is necessary to document that similar populations of cells are being compared. The purpose of the current study was to determine whether these non-T-cells alter the lymphocyte response of burn patients to mitogenic and antigenic stimuli.

Materials and Methods

Patient Population

Thirteen patients, eight men and five women, were studied. The mean age of these patients was 32 ± 13 years, and the mean total body surface area burn size was 32% ± 17%, with a third-degree component of 12% ± 11%. One patient died, for a mortality rate of 8%. In order to determine the influence of thermal injury on cell function in the abscence of sepsis, blood samples were not obtained from patients while they were septic. Similarly, since sur-

gery and anesthesia may influence cell function, blood samples were not collected until at least 48 hours after the operation.

All patients were treated with a standard therapeutic regimen, which was modified according to the clinical response of each patient. The protocol consisted of fluid resuscitation based on the Parkland Formula, topical antimicrobial agent administration consisting of sulfadiazine silver or mafenide acetate or both, plus nutritional support based on the 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 except in the immediate perioperative period, with one dose administered before and one dose after the operation. All deep partial- and full-thickness burns were excised and grafted as soon as the patients were hemodynamically stable. The initial operation was usually performed by the fifth postburn day.

Cell Preparations

Fifteen milliliters of blood were drawn from the patients at various times postburn and anticoagulated with either heparin or ethylenediaminetetracetic acid (EDTA). The blood was layered over a ficoll-hypaque gradient and centrifuged at 400 × *g* for 30 minutes.[14] The mononuclear layer was removed and adjusted to a concentration of 4 × 10^6 cells/ml in a 20% solution of heat-inactivated human AB serum in minimal essential medium (MEM) to which penicillin (10,000 units/ml) and streptomycin (10,000 μg/ml) had been added. Viability was tested by eosin red dye and was greater than 95%. Mononuclear cells obtained from healthy adult volunteers were processed in the same fashion and served as daily control cells.

Spontaneous Blastogenic Transformation (SBT) Assay[15]

An aliquot (50 μl) of the control or patient mononuclear cell suspension containing 2 × 10^5 cells was placed into individual wells of microtiter plates. The cell cultures were immediately pulsed with 1 μCi of tritiated thymidine and incubated for 15 hours at 37 C in a 5% CO_2 environment. The cell cultures were then 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.

Lymphocyte (Mitogen) Blastogenic Assay[13]

Aliquots of the mononuclear cell preparation containing 2 × 10^5 cells were placed into wells of a microtiter plate. The cells were incubated with 0 or 5 μg per well of the mitogen PHA at 37 C in 5% CO_2. On the third day, the cell cultures were pulsed with 1 μCi of tritiated thymidine and incubated an additional 18 hours. The cells were harvested as described for the SBT. All assays were performed in triplicate and the results averaged.

Mixed Lymphocyte Culture Assay[16]

Aliquots of the control and patient's mononuclear cell preperation were divided into responding and stimulating cell populations. The stimulator cells were incubated with mitomycin (25 μg per 2 × 10^6 cells) for 30 min at 37 C. After the incubation was completed, the mitomycin-treated cells were washed three times and resuspended in MEM containing 20% human AB serum. In the allogeneic mixed lymphocyte cultures, allogeneic stimulating cells were used, while in the autologous mixed lymphocyte culture assay, autologous stimulating cells were used. Each culture contained 2 × 10^5 responding and 2 × 10^5 stimulating cells per well. The cells were cultured for 5 days at 37 C in 5% CO_2. On the fifth day, 1 μCi of tritiated thymidine was added to each well, and 18 hours later, the cells were harvested and thymidine incorporation determined. All assays were performed in triplicate and the results averaged.

Isolation of Rosetting and Nonrosetting Cells[17]

Ficoll-hypaque isolated leukocytes adjusted to a final concentration of 2 × 10^6 cells/ml were incubated with an equal volume of 1% AET-treated sheep red blood cells (S-RBCs) at 37 C for 15 minutes. The AET-treated S-RBCs were produced by incubated washed S-RBCs (0.5 ml) with 2 ml of 0.143 M AET (2-S-aminoethylisothiouronium bromide hydrobromide) (Sigma Chemical Co., St. Louis, MO) at a pH of 9. After incubation (15 minutes at 37 C), the S-RBCs were washed and adjusted to a final concentration of 1% in Roswell Park Media (RPMI)-1640 containing 10% heat-inactivated fetal calf serum. The leukocyte/S-RBC mixture was then centrifuged at 200 × *g* for 5 minutes and the pelleted cells incubated for 45 minutes at 4 C. To seperate the rosetting from nonresetting cells, the leukocyte/S-RBC suspension was layered over 5 ml of ficoll-hypaque and centrifuged at 400 × *g* for 30 minutes. The nonrosetting cells were removed from the ficoll-hypaque interface and washed twice with media and resuspended to a final concentration of 4 × 10^6 cells/ml in MEM containing 20% heat-inactivated human AB serum. The rosetting cells, which sedimented to the bottom of the ficoll-hypaque preperation, were collected and the S-RBCs lysed with distilled water. The rosetting cells were then washed twice with media and resuspended in MEM containing 20% heat-inactivated human AB serum at a final concentration of 4 × 10^6 cells/ml.

TABLE 1. *The Levels of Spontaneous (SBT) and Mitogen-Stimulated (PHA) Blastogenesis Is Different Between Burn Patients and Controls (All Postburn Days Combined)*

	SBT (DPM)				PHA Stimulated (DPM)			
Group	*N*	Unfractionated Ficoll-Hypaque	Nonrosette-forming Cells	S-RBC Rosette-forming Cells	*N*	Unfractionated Ficoll-Hypaque	Nonrosette-forming Cells	S-RBC Rosette-forming Cells
Control	65	407 ± 17	307 ± 36*	336 ± 16*	60	77,038 ± 3,613	29,438 ± 2,091†	76,790 ± 4,478
Patient	65	9,141 ± 1,093	12,320 ± 1,579*	3,050 ± 355*	60	55,689 ± 6,395	6,744 ± 1,832†	84,012 ± 5,670‡
p (ANOVA)		<0.01	<0.01	<0.01		<0.01	<0.05	NS§

All values expressed as mean ± SEM.
* $p < 0.05$ versus ficoll-hypaque whole cell population.
† $p < 0.01$ versus ficoll-hypaque whole cell and rosetted cell populations.
‡ $p < 0.01$ versus ficoll-hypaque whole cell population.
§ NS = not significant.

Flow Cytometric Analysis

Ficoll-hypaque and or S-RBC purified mononuclear leukocyte populations were incubated with monoclonal antibodies for 30 minutes at 4 C. The cells were then washed twice and reacted with purified, fluorescein-labeled anti-mouse IgG. The fluorescein-labeled cells were analyzed on an Epics C flow cytometer (Coulter Electronics, Hialeah, FL) equiped with a 2-watt argon laser. The argon laser was set on the 488-nm line and run at 500 mW. Calibration was performed using fluorescent 10-μm polystyrene beads (Coulter Electronics). For each sample, a minimum of 5000 cells was analyzed, and the number of cells labeled by monoclonal antibodies specific for pan-T-lymphocytes (OKT3) was determined. Nonspecific monoclonal antibody binding was determined by running a negative control with mouse IgG. Forward angle light scatter (FALS) and log 90-degree light scatter (L90LS) were used to identify and gate on specific leukocyte populations.

Statistics

Comparisons between multiple groups were made by analysis of variance. A two-tailed Student's *t*-test was used to compare two groups, when appropriate.

Results

The rate of spontaneous uptake of ^{3}H-thymidine by ficoll-hypaque purified leukocytes was significantly higher in the leukocytes isolated from burn patients than from healthy controls. In contrast, the level of mitogen-stimulated ^{3}H-thymidine uptake by the patients' leukocytes was reduced (Table 1). The decrease in mitogen-stimulated blastogenesis observed in the ficoll-hypaque purified leukocytes from the burn patients may be an artifact, since ficoll-hypaque purified cells from burn patients are contaminated with large numbers of immature nonlymphoid cells that do not respond to lymphocyte-dependent stimuli, such as the mitogen PHA. Thus, the ficoll-hypaque purified cells were separated into T-cell enriched and T-cell poor populations by S-RBC rosetting and the SBT and mitogen response of each cell population determined (Table 1). The leukocytes from the controls that formed S-RBC rosettes (T-cell enriched) responded to PHA in a similar fashion to the unfractionated ficoll-hypaque cell population, although the SBT of the rosetted cells decreased (Table 1). Both the SBT and mitogen response of the nonrosetting (T-cell poor) fraction of the control cells was less than the unfractionated, whole cell population. The pattern found when the patients cells were separated into T-cell rich and poor fractions differed from the control cells in a number of ways. The nonrosetting patient cells had the highest levels of SBT activity and the lowest blastogenic response to PHA, while the rosetted cells responded best to PHA and had the lowest SBT levels. The patients' T-cell rich fraction responded normally to PHA, indicating that the impaired mitogenic response of the unfractionated ficoll-hypaque whole cell population may not be due to impaired lymphocyte function but instead may be due to decreased lymphocyte numbers.

Since lymphocyte function may change during the hospital course, the results reported in Table 1 were analyzed according to the day postburn the cells were tested. The SBT of the patients' unfractionated ficoll-hypaque purified cells were higher than the control cells at all times postburn (Table 2). Just as shown in Table 1, the majority of the cells that spontaneously took up ^{3}H-thymidine were in the fraction of cells that did not bind to S-RBCs and thus appear to be functionally non-T cells. Although the SBT of the S-RBC rosetting patient cells were only 20–30% of the SBT of the nonrosetted cells, the levels of spontaneous ^{3}H-thymidine uptake of the patients' rosetted cells were significantly higher than control rosetted cells from the fourth postburn day on. These results suggest that both T cells and non-T cells are activated postburn, even in the absence of sepsis.

The mitogen response of the patients' unfractionated ficoll-hypaque purified cells was significantly depressed for the first 2 weeks postburn, then returned to normal

TABLE 2. *Comparison Between SBT of Patient Versus Control Leukocyte Populations According to Postburn Day*

	Postburn Day					
	1–3	4–7	8–14	15–21	22–28	29 On
Unfractionated Ficoll-Hypaque cells						
Control	395 ± 36	372 ± 53	434 ± 45	391 ± 26	394 ± 26	472 ± 66
Patient	1,523 ± 443	10,480 ± 3,494	15,205 ± 2,323	5,784 ± 1,441	10,913 ± 3,183	9,325 ± 1,649
p (ANOVA)	<0.05	<0.05	<0.01	<0.01	<0.01	<0.01
Nonrosette-forming cells						
Control	255 ± 41	253 ± 44	292 ± 38	407 ± 163	284 ± 33	349 ± 70
Patient	1,732 ± 652	12,586 ± 4,026	20,286 ± 3,687	9,251 ± 2,671	16,831 ± 4,889	9,932 ± 2,384
p (ANOVA)	<0.05	<0.01	<0.01	<0.01	<0.01	<0.01
S-RBC rosette-forming cells						
Control	305 ± 40	241 ± 20	348 ± 30	359 ± 32	364 ± 47	430 ± 70
Patient	817 ± 277	3,400 ± 1,144	4,123 ± 789	2,589 ± 594	3,811 ± 1,119	3,556 ± 792
p (ANOVA)	<0.08	<0.05	<0.01	<0.01	<0.05	<0.01

All values expressed as mean ± SEM.

(Table 3). However, the mitogen response of the S-RBC rosetting fraction was normal at all times postburn, indicating that after thermal injury the decreased PHA response may not be due to impaired T-cell function, but instead due to the presence of large numbers of nonlymphoid cells contaminating the ficoll-hypaque preparation.

The rosetting procedure did not appear to activate the control mononuclear cells, since the mitogenic response of the unfractionated ficoll-hypaque and S-RBC rosetted cells were similar. In contrast, the patients' rosetted lymphocytes had a significantly higher mitogen-stimulated blastogenic response than their unfractionated cells on days 1–3 and 8–14 postburn, a time when the mitogen response of the unfractionated cells was depressed. On the other hand, from postburn day 15 on, when the mitogenic response of the unfractionated cells had returned to normal, there was no difference between the mitogen response of the rosetted and unfractionated patient cell populations (Table 3). Similar results were documented when the stimulation indexes of the patient and control unfractionated, S-RBC rosetted, and nonrosetted cells were compared (data not shown).

The rosetting technique did not appear to totally seperate cells into mitogen responsive and unresponsive groups, since the nonrosetting cell fraction in the controls had significant levels of ^{3}H-thymidine uptake after mitogen stimulation (Table 3). Even so, the nonrosetting patient cells did not respond to PHA as well as the control nonrosetting cells. Since the patients cells that formed S-RBC rosettes responded normally to PHA, while the nonrosetting cells did not, it appears that the impaired mitogenic response documented in the ficoll-hypaque purified leukocytes was at least partly due to the presence of the nonrosetting cells.

Quantitation of the number of leukocytes that did or did not form S-RBC rosettes was determined for both the control and patient populations. The mean leukocyte recovery after ficoll-hypaque purification was higher in the

TABLE 3. *Comparison Between Mitogen Induced Blastogenesis of Patient Versus Control Leukocyte Populations According to Postburn Day*

	Postburn Day				
	1–3	4–7	8–14	15–21	22 On
Unfractionated Ficoll-Hypaque cells					
Control	90,231 ± 7,666	73,755 ± 9,576	75,573 ± 7,861	79,352 ± 8,657	70,451 ± 6,904
Patient	58,215 ± 13,018	36,945 ± 6,037	49,792 ± 11,678	72,329 ± 17,526	56,502 ± 15,390
p (ANOVA)	<0.01	<0.05	<0.05	NS	NS
Nonrosette-forming cells					
Control	38,327 ± 7,383	24,455 ± 3,298	32,762 ± 4,191	28,893 ± 4,772	24,466 ± 3,498
Patient	7,381 ± 3,473	2,767 ± 1,446	3,391 ± 740	7,661 ± 10,101	11,083 ± 6,534
P (ANOVA)	<0.01	<0.05	<0.05	<0.01	NS
S-RBC rosette-forming cells					
Control	74,517 ± 7,928	66,772 ± 10,735	80,037 ± 8,649	70,988 ± 9,721	86,162 ± 11,344
Patient	87,834 ± 12,066*	56,760 ± 11,747	93,104 ± 14,783*	95,364 ± 12,955	79,747 ± 9,160
P (ANOVA)	NS	NS	NS	NS	NS

All values expressed as mean SEM.

* $p < 0.05$ versus patient ficoll-hypaque cells.

TABLE 4. *Quantitation of Leukocyte Yields After Ficoll-Hypaque (FH) Purification and Subsequent Separation of S-RBC Rosette-Forming (R) from Nonrosette-Forming (NR) Leukocytes*

Group	N	Total Leukocytes (FH)	NR Forming	SRBC-R Forming	Cells Required* After Rosetting (%)	Group	N	% SRBC† Forming Cells	% OKT3 Positive N	% SRBC-R Forming	% NR Forming
Control	65	31.2 ± 9.1	8.1 ± 2.5	10.9 ± 3.1	62 ± 8	Control	65	58 ± 4	51	79 ± 8	21 ± 5
Patient	65	39.9 ± 14	15.4 ± 8.0	8.2 ± 3.5	60 ± 11	Patient	65	37 ± 13	51	84 ± 11	15 ± 5
P		<0.01	<0.01	<0.05	NS	P		<0.01		<0.01	<0.01

All values shown are mean ± SD; cell count × 10^6.

* Per cent of cell recovered = $\frac{NR + R}{FH} \times 100$.

† Per cent of cells recovered that formed S-RBC rosettes = $\frac{R}{NR + R} \times 100$.

patients than the controls ($p < 0.01$) (Table 4), reflecting the fact that leukocytosis is common after thermal injury. Although the patients had higher total numbers of leukocytes isolated from their whole blood, fewer of the patients leukocytes formed S-RBC rosettes than control leukocytes ($p < 0.05$). This was true whether the absolute number or percentage (58% *vs.* 37%) of rosette forming cells was compared (Table 4). At all times postburn, the percentage of leukocytes forming S-RBC rosettes was lower in the patients than the controls ($p < 0.01$) (data not shown). The decrease in the numbers of patient leukocytes forming S-RBC rosettes was not due to a decrease in the percentage of patient versus control cells recovered after rosetting. The percent recovery of the control cells (62% ± 8%) was not different from the percentage of patient cells recovered (60% ± 11%). Thus, it appears that although the patients had more leukocytes recovered by ficoll-hypaque purification than did the controls, the percentage of these cells that were functionally mature T cells, as judged by S-RBC rosetting, was lower. These results of decreased numbers of functional T cells, based on S-RBC rosetting, are consistent with their decreased functional ability to respond to the mitogen PHA.

The pan-T-cell marker OKT3 was used to quantitate the percentage of T cells in the rosette-forming and nonrosette-forming populations. When the patients were stratified according to postburn day, the percentage of OKT3-positive cells between the patient and control leukocyte rosette-forming populations was not statistically different (data not shown), although, when all the days were combined, the percentage of OKT3-positive cells was higher in the rosetted cell population of the patients than the controls ($p < 0.01$) (Table 4). In contrast, the percent of OKT3-positive cells in the nonrosette-forming leukocyte population was lower in the patients than the controls ($p < 0.01$) (Table 4).

The presence of OKT3-positive lymphocytes in the nonrosette cell population appears to explain why the nonrosette-forming cell populations could be stimulated by PHA to take up ^{3}H-thymidine. Although the percentage of OKT3-positive patient cells (15% ± 5%) was 72% of the control cell level (21% ± 5%), the mitogen response

TABLE 5. *Allogeneic MLC of Ficoll-Hypaque Purified Leukocytes from Burn Patients but not Controls Is Increased*

	Postburn Day					
	All Days	1–3	4–7	8–14	15–21	22 On
Unfractionated Ficoll-Hypaque cells						
Control	5,727 ± 300	6,377 ± 704	3,936 ± 507	5,993 ± 417	5,312 ± 532	6,672 ± 894
Patient	4,115 ± 753	2,766 ± 503	2,857 ± 1,469	4,633 ± 1,919	5,681 ± 2,330	4,149 ± 1,361
p (ANOVA)	<0.05	<0.01	NS	NS	NS	NS
Nonrosette-forming cells						
Control	2,278 ± 91	2,365 ± 234	2,121 ± 192	2,052 ± 116	2,182 ± 127	2,690 ± 295
Patient	717 ± 136	444 ± 107	749 ± 480	561 ± 211	1,134 ± 433	710 ± 224
p (ANOVA)	<0.01	<0.01	<0.01	<0.01	NS	<0.01
S-RBC rosette-forming cells						
Control	6,579 ± 358*	7,851 ± 862	5,126 ± 519	6,962 ± 458	6,380 ± 728	6,338 ± 1,174
Patient	8,937 ± 1,684†	5,195 ± 1,737	5,825 ± 2,595	12,796 ± 4,869	10,659 ± 4,564	8,442 ± 2,716
p (ANOVA)	<0.05	NS	NS	NS	NS	NS

All values expressed as mean ± SEM. Total number of assays is 49, with 8–12 assays performed during each postburn time period.

* NS versus control whole cell population.

† $p < 0.01$ versus patient's whole cell population.

of the patients' cells (6744 cpm) was only 23% of the control nonrosetted cells (29,438 cpm) (Table 1). These results raise two possibilities: either that the nonrosetted patient OKT3-positive lymphocytes were less responsive to mitogen stimulation than control nonrosetting OKT3-positive cells, or that the nonlymphoid cells in the nonrosetting patient population were inhibiting the OKT3-positive lymphocytes from responding to PHA. Unfortunately, which of these possibilities is true cannot be answered from the current study.

The ability of the various leukocyte fractions to respond to antigenic stimuli was measured in an allogeneic mixed lymphocyte culture (MLC) assay. When all the assays were combined, the allogeneic MLC response of the unfractionated ficoll-hypaque purified leukocytes from the burn patients was lower than that observed in the control population ($p < 0.05$). In contrast, the MLC response of the patients rosetted cells was higher ($p < 0.05$) (Table 5; all days). The MLC response of the patients leukocytes, but not the controls, was increased in the rosetted fraction compared to the whole cell population (Fig. 1). Thus, the patients leukocyte fractions responded to antigens (MLC response) and mitogens (PHA) in a similar fashion.

When the patients' leukocyte response in the allogeneic MLC was analyzed according to postburn day, only during the first 3 days postburn was the patients' unfractionated leukocyte response depressed. Failure to document a significant depression from day 4 on may relate to an insufficient number of specimens being studied during these specific periods. The MLC response of the patients' nonrosette-forming cells was depressed during the entire postburn course, except for the period from the 15th to the 21st postburn day (Table 5). Although the MLC response of the patients' rosetted cells was generally higher than the control cells at most periods postburn, these differences did not reach statistical significance.

In contrast to the allogeneic MLC response, the patients' autologous MLC could not be restored to normal by seperating the rosette from the nonrosette-forming cells (Table 6). Furthermore, the patients' autologous MLC response was impaired throughout the hospital course in both the whole cell and nonrosette-forming cell populations. Thus, it appears that the response of all fractions of the patients leukocytes to self-antigens may be impaired after thermal injury, although the T-cell rich fraction of these same cells will respond normally to the antigenic and mitogenic stimuli tested in this study.

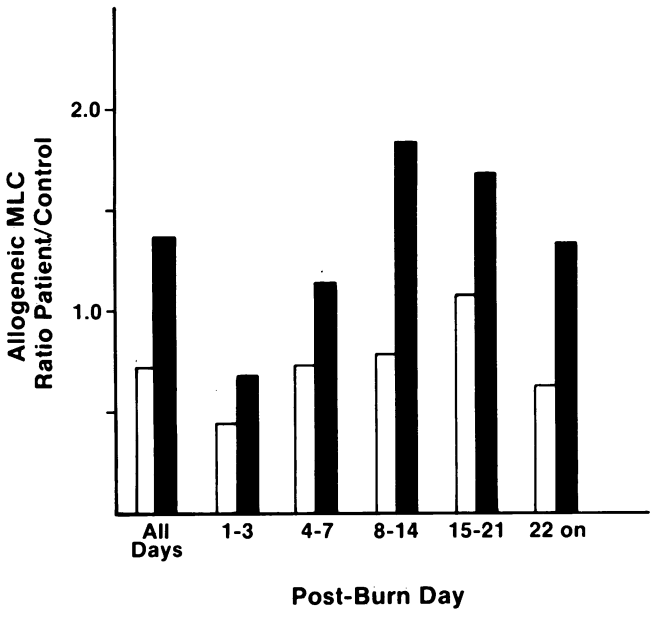

FIG. 1. The response of the patients' ficoll-hypaque purified cells (open bars) in the allogeneic MLC was depressed, while the response of the S-RBC purified T cells was normal or increased (closed bars).

Discussion

Although it is generally accepted that cell-mediated immunity is impaired after thermal injury, there is no

TABLE 6. *Comparison of Autologous MLC Between Control and Burn Patients Subpopulations*

	Postburn Day					
	All Days	1–3	4–7	8–14	15–21	22 On
Unfractionated ficoll-hypaque cells						
Control	647 ± 21	733 ± 42	585 ± 51	596 ± 37	613 ± 60	726 ± 24
Patient	321 ± 35	378 ± 92	254 ± 48	328 ± 97	342 ± 70	293 ± 64
p (ANOVA)	<0.01	<0.01	<0.05	<0.05	<0.01	<0.01
Nonrosette-forming cells						
Control	388 ± 17	410 ± 44	311 ± 40	427 ± 38	414 ± 36	355 ± 24
Patient	152 ± 14	164 ± 37	115 ± 15	139 ± 30	185 ± 33	154 ± 32
p (ANOVA)	<0.01	<0.01	<0.05	<0.01	<0.05	<0.01
S-RBC rosette-forming cells						
Control	648 ± 22	637 ± 60	618 ± 64	622 ± 44	689 ± 53	676 ± 25
Patient	410 ± 46	548 ± 94	494 ± 147	431 ± 95	355 ± 94	236 ± 70
p (ANOVA)	<0.01	NS	NS	NS	<0.01	<0.01

All values expressed as mean ± SEM. Total number or assays is 48, with 8–12 assays performed during each time period.

consensus on whether *in vitro* cell-mediated immunity is normal,[11] increased,[18,19] or decreased.[20,21] Furthermore, controversy exists over whether changes in the cellular response to lymphocyte stimuli, such as PHA, correlate prognostically with the onset of sepsis or death.[11,20,22,23] To some extent, these differences appear to be related to differences in patient care protocols, patient selection, and data analysis. However, another reason for these conflicting results may be that the cell populations being studied are not homogeneous and contain nonlymphoid cells.

A potential risk of all standardized *in vitro* assays is that the results of the assay may not accurately reflect the complex interactions that are occurring *in vivo*. Therefore, several years ago, we measured the level of *in vivo* lymphocyte activity, as reflected by the SBT, together with the ability of these same cells to respond to the mitogen PHA in 29 burn victims.[13] The two major conclusions of the study were (1) that the SBT activity of the patients cells was increased, even when the mitogen response was depressed, and (2) that changes in the SBT, but not the mitogen response, predicted sepsis. Based on the results of this work, we postulated that *in vitro* postburn impaired cell-mediated immunity may not be due to impaired (lazy) lymphocytes but due to the fact that these cells were activated *in vivo*.[13]

In subsequent experiments performed to clarify why the SBT was increased, even when mitogen-stimulated blastogenesis was impaired, we discovered that ficoll-hypaque purified mononuclear cell preparations from burn patients contained a large number of metabolically active leukocytes that were not mature T lymphocytes or macrophages (12). Since these non-T cells took up thymidine but did not respond to PHA, these metabolically active leukocytes could alter the results of *in vitro* lymphocyte function tests. To test this possibility, we serially evaluated the response of several fractions of burn patient leukocytes to mitogenic and antigenic stimuli. The mitogen and alloantigen responses of S-RBC purified T cells from the burn patients were normal, even when the response of unfractionated ficoll-hypaque whole cell leukocyte preperations were depressed. Thus, it appears that lymphocytes from burn patients, during nonseptic periods, respond normally. Whether the decreased response of these cells, when tested in the presence of nonrosetting cells, is due to decreased numbers of lymphocytes or to inhibition of lymphocyte function by the nonrosetting cells cannot be determined from this study.

In contrast to our results with PHA and alloantigens, the burn patients' lymphocytes had a decreased response to autoantigens, as measured by the autologous MLC. This decreased response was not corrected by removing the nonrosetting cells from the whole cell population. The biologic and clinical significance of a depressed autoantigen response, in the face of normal mitogen and alloantigen responses, cannot be determined from the results of this study. However, it is tempting to speculate that this pattern of lymphocyte reactivity could be of benefit, since the patient would be less likely to respond to altered (exposed) self-antigens, while maintaining an intact immune response to foreign antigens. Further study is clearly needed to resolve this and other potential explanations for these observations.

The observation that ficoll-hypaque purified mononuclear cells were contaminated by large numbers of nonlymphoid cells was first made by Volenec et al. in 1979.[24] Since that time, we[12] and others[25] have observed that density purification techniques do not seperate mature mononuclear cells from other cell populations in the burn patient. However, this is the first reported study to evaluate whether these contaminating cells could influence the results of *in vitro* assays of lymphocyte function. The results of this study clearly document that lymphocytes respond normally to PHA and alloantigens after the contaminating cell population has been removed. We believe that future studies should be carried out to better define the influence of the nonrosetting cells on lymphocyte function, as well as studies evaluating the immune responsiveness and immune regulatory functions of the rosette-forming T cells. By studying more purified cell populations, it should be potentially possible to resolve some of the controversies about the effect of a thermal injury on cell-mediated immunity.

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