Image Cover Sheet

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TITLE

MECHANISMS OF IMMUNE FAILURE IN BURN INJURY

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MECHANISMS OF IMMUNE FAILURE IN BURN INJURY

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Summary

// The burden on military medical services in handling burn casualties is daunting as all physiological systems will become affected. Severe burns in a battlefield setting have a very low salvage rate, to a great degree because of the immune failure which invariably develops. Evaluations of responses of lymphocytes taken from burn patients over several weeks following the burn (> 30% TBSA), have revealed that the immune failure which follows thermal injury involves T cell activation events. Interleukin 2, which is normally produced by activated T lymphocytes, is very poorly produced by cells cultivated in vitro taken from non-surviving patients, whereas some production continues, although at below normal levels, in patients who ultimately survive their injury. //

IL2 exogenously added to lymphocyte cultures enhances the proliferation of cells from surviving patients but gives no such help to cells from non-survivors. The TAC portion of the IL2 receptor (IL2Rα), expressed on the T cell surface, appears to be responsible for this difference, as the number of lymphocytes able to express IL2Rα falls post-burn. A lipid protein complex (LPC) produced in skin by burning has been shown to inhibit the immune response in vivo and the growth of IL2-dependent lymphocytes in culture. Cerium nitrate, applied topically to the burn patient, is thought to fix the LPC in the burn eschar and prevent its entry into the circulation. In a study of 10 patients, bathed in cerium nitrate, some T lymphocyte activities were found to be in the normal range rather than suppressed. Such a treatment promises to be useful in improving chances of survival in severe burn injury.

Introduction

For military medical services the burn casualty poses enormous logistic problems, since the burn, as a form of trauma, evokes to an exaggerated degree, all of the systemic responses seen in other injured patients. An extensive burn makes the patient with such an injury the universal trauma model. Not only is there extensive skin damage but potentially every organ system can become affected. It follows that all treatment procedures applied to patients, and information gathered from studies of burned patients, have application also to all other trauma patients (24). Hence, research into burn injury is actively supported in many world centres and any understanding which leads to novel approaches to treatment may reduce the burden of care universally.

Unlike mechanical injury the burn induces quantitative changes in relation to the magnitude of the injury, and all systems may become affected in time, after a severe burn injury (19). Reduction of early death due to shock and acute renal failure achieved by fluid resuscitation, has only revealed later, other previously obscured problems. Once out of the critical stage severely burned patients typically fluctuate from serious to critical condition several times as respiratory, cardiovascular and infectious complications develop. In a military setting stabilization of the patient may be the initial challenge, but appropriate longer term care of the patient has to be modified according to the service in the available time frame. On the assumption that problems of casualty sorting, of evaluation, and first treatment techniques are optimized, the major problem for the burn casualty then is the ultimately higher than normal risk of late death simply due to delay in achieving high level critical care.

Very early fluid resuscitation may appear to be critical for survival, but only in the short term. This was demonstrated by the disaster at the *Los Alfaques* campground in Spain where a tanker truck, carrying a volatile liquid fuel, crashed on the roadside at the camp. The fuel's expanding vapour engulfed 242 campers in a flaming explosion. The truck blocked the road such that of the 140 immediate survivors 58 were evacuated north to Barcelona and 82 south to Valencia. Only the northbound group received fluids en route, from small local hospitals. Thus, as the *Los Alfaques* disaster has revealed (3), by having two comparable groups of burn victims of 85.5 ± 19.5 % total burn surface area (TBSA) and 81.2 ± 24.0 %TBSA, with ages of 26.3 ± 17.0 and 28.0 ± 16.2 respectively, early fluid resuscitation in only the first group, permitted greater survival for the first few weeks but no significant difference between groups after two months (21% and 28% surviving) (Figure 1). It is for these reasons that heroic first attempts to treat the severely burned, with existing procedures, under wartime conditions, will have doubtful long term consequences. This fact underlies policies for treatment on the battlefield.

With increasing numbers of burn casualties in warfare (Table 1) the burden on field medical services becomes daunting. In anticipation of these events some NATO countries have defined their policy for choosing which burn cases will be treated. Whereas a young healthy adult burn patient, in a civilian setting, will have a risk of survival of 0.5, if his lesions represent 60% TBSA (5), there have been policies stating that casualties with burns

over 40% are "beyond the scope of therapeutic capabilities in times of crisis" (8). These thresholds have been supported by the indication that with burns over 60% TBSA the salvage rate of military patients has been low and survival could not be assured (32).

The main cause of death after severe burn injury has been listed as sepsis (25), which most often is accompanied by multiple organ failure (MOF). However, the apparent lack of resistance of the burn patient indicates a "host problem" rather than a problem of increased virulence of the invading microorganism. Furthermore, in the late stages of burns circulating bacteria cannot be detected in up to half the patients who die from what appears to be sepsis (9). Hence the MOF is thought to be due to devitalized tissue activating phagocytic and other cells to secrete nefarious mediators which dysregulate homeostasis.

A significant effect of these burn-induced mediators is on host defences, and this in particular involves first the destruction of the skin barrier, the impairment of tissue blood flow, the depression of the immune response, and then the secondary effects of therapy and complications. Implications of cellular immune response failure (T lymphocyte failure) had been suggested by observations in burn injury, noted over 4 decades ago, of prolonged allograft survival in skin grafting procedures (4). Graft rejection represents activity of the T lymphocyte compartment of the immune response, and other T cell functions were also noticed to have failed following burn injury, such as the delayed type hypersensitivity (DTH) response (23), the proliferative response of lymphocytes to a T cell mitogen (7), the cytolytic response (16), and the mixed lymphocyte response (20) (Table 2).

Immune Failure in Burn Injury.

In 1984 the Defence & Civil Institute of Environmental Medicine initiated an investigation into the mechanisms of immune failure in burn injury, through the Ross Tilley Burn Centre at the Wellesley Hospital, Toronto.

To study T cell activity of burn patients, peripheral blood mononuclear cells (PBMC), which represent 27% of the leukocytes (Table 3), were taken every week or ten days over the course of hospitalization, and *in vitro* lymphocyte IL2 production was assessed by culturing them with mitogen and assaying the culture supernatants for IL2 by the proliferative response of an IL2-dependent cell line (36). IL2 production was below normal, and both non-survivors and survivors could not be differentiated on the day of the burn. However, over the hospitalization period, *in vitro* IL2 secretion levels increased closer to normal levels in survivors, but decreased to undetectable levels in non-survivors (Figure 2).

An assay of the PBMC proliferative response in the presence of exogenous IL2 is shown in Figure 3. Low responses to IL2 on day 1, and a week later, were common to all patients. However, survivors' responses recovered by the third week in contrast to those of the others. Once again a marked difference was observed between survivors and non-survivors with regards to IL2 activity in the burn (37).

Survivors' PBMC cultivated *in vitro* 3 days with mitogen, displayed a high level of the surface α receptor for IL2 (IL2Rα or TAC) when cells were taken on the day of the burn. Figure 4 shows that at some point in the second or third week the IL2Rα levels would drop, but later return, in survivors, to day 1 levels. The lowest point may be Day 10 or 20 or both, as shown for 3 selected patients (Figure 4). In non-survivors the cell surface IL2Rα would steadily decrease from Day 1 to the time of death (36) (Figure 4). Overall results from a large group of patients showed that during the course of hospitalization, IL2Rα would be expressed on cells at the time of the lowest levels observed, at a mean level of about 50% of the surviving individuals' first day levels, but at virtually undetectable levels for non-survivors (Figure 5). Low levels of *in vitro* lymphocyte IL2 production and low levels of the α IL2 receptor, inducible *in vitro* on the surface of the lymphocytes, appeared therefore to be critical indices for non-survival, and suggested problems with the T cell activation mechanism.

Burn-Induced Eschar Toxic Products.

For over a century (39) researchers have believed that burn injury causes the production and/or release of mediators which have toxic or suppressive effects on normal physiological functions. Indeed, many T cell functions are inhibited by factors circulating in the serum (Table 2). Present knowledge of the cytokine cascade and its relationship to prostaglandin activity may provide ready explanations for the inhibition of any particular function, as the cytokines (e.g the interleukins) serve to mediate a regulatory system within the immune response, consisting of specific ligands and cell membrane receptors. As we have seen, it is this cytokine system which has clearly become dysregulated in burn injury, more so in non-survivors than in survivors.

Quantitative differences in burn immunosuppression were found to bear a clear relationship with quantity and type of burn tissue. Mice implanted with increasing amounts of burned skin (eschar) (but not burned liver, for example), were increasingly immunosuppressed (10). The quantitative toxicity of burned skin has also been shown to be related to the burn temperature (1). Consistent with these two findings, in the burned mouse, immediate excision of the eschar avoided immunosuppression (11). Beneficial effects of prompt eschar excision have also been demonstrated in humans, particularly in burned children (6,38) and young adults (12). Conclusions to the contrary (15) depend on how early the excision is made, for

burned skin has been shown to contain a toxic product (1,2) which can appear in the circulation within a day of the burn injury (33). This particular eschar product is not a tissue breakdown product but a heat-induced cross-linking of a complex of six skin cell membrane lipid-associated proteins (30). They are combined to form a lipid protein complex (LPC), of about 3 million kDa, which was shown to damage cell ultrastructure in the same fashion as does burn injury (13). LPC also disturbed cell metabolic function by its effect on mitochondrial membranes (29). Altogether many effects of the LPC have been found to mimic the various consequences of burn injury (14), and these even include immunosuppression. In vivo, LPC enhanced the mouse susceptibility to pseudomonas infection (31), and the mouse immune response to sheep erythrocytes (Figure 6), being 1000 times more immunosuppressive than bacterial endotoxins on a molar basis (34). It was also inhibitory of the T lymphocyte proliferative response to PHA *in vitro* (21), and inhibited PWM-induced IgG production (35) as well as the growth of IL2-dependent cells in culture (34) (Figure 7). Thus, it is implicated in the very mechanism, T cell activation, which appears to be critical to surviving major burns.

Use of topical cerium nitrate in burn treatment.

Serving as a topical antiseptic for burn wounds silver nitrate had been used extensively since 1945. However, it blackened the bed clothes and affected electrolyte balance of the patients adversely. Sulfamylon was also introduced as a topical agent but it also had drawbacks. Alternatives were sought and cerium nitrate and silver sulfadiazine (SSD) were identified. When assays for antibacterial activity were performed with these two agents SSD demonstrated superior antibacterial activity in *in vitro* tests on bacterial plate cultures, and thus rose to prominence. Its use is nearly universal today. However, in clinical trials on burn patients, Monafo, using topical cerium nitrate solution on gauze dressings, observed an unusually high survival rate among patients whose burns warranted a prediction of a lower survival rate (17,18) (Tables 4, 5). Cerium particularly minimized the incidences of late death in burn victims with larger %TBSA, yet clinically it appeared not to be a superior topical antiseptic, confirming the *in vitro* comparisons. However, its weaker antiseptic value is nevertheless useful in synergism with SSD (27).

The use of cerium in treating burns has not yet been widely accepted and is still at the experimental stage. However it became appreciated that the reports of improved clinical outcome from use of topical cerium might be explained by the local destruction of the toxic lipid protein complex in the burn eschar. Studies on the binding capability of cerium with the complex revealed a high affinity of the two with consequent denaturation of the LPC (13). Therefore it was reasoned that one bathing, long enough to allow for good contact of eschar toxic complex with cerium nitrate, should inhibit LPC resorption from the burned tissue into the circulation, effectively inducing a "chemical excision" of the eschar's dangerous properties. Indeed, experiments had confirmed that topical application of cerium nitrate to scalded skin of mice prevented the T lymphocyte failure common to the conventionally treated burns (22).

Based on the binding affinity of cerium for the eschar toxic complex, the intensive care unit of the Kantonsspital, Basel, Switzerland, has, for a number of years, used a treatment routine for burn patients which involves one tubbing only at the time of admission, in 0.04M cerium nitrate. The incidence of survival in large burns has been found to be exceptionally high (28) with 9 out of 10 patients surviving risks of mortality (calculated according to *Roi et al. (26)*) greater than 0.8 (Figure 8). A risk of 0.8 implies that 8 out of 10 patients with such complications should die, yet several survived with risks greater than 3 patients who did die, of particular complications not strictly burn related. With lower risk patients, who might all be expected to survive, the value of cerium is not revealed by checking survival or death. However if cerium binds to the LPC in the eschar and LPC is a key agent initiating immune dysregulation, the parameters of the immune response should be improved in patients who receive cerium treatment.

A group of patients was therefore bathed once in 0.04M cerium nitrate and their PBMC were taken at ten day intervals for an assessment of lymphocyte surface IL2Rα induction and of IL2 secretion, in response to mitogens *in vitro*. Compared to former burn survivors whose lymphocytes displayed, at the time of lowest immune responses, a surface IL2Rα level around 50% of the first day level (Figure 5), the cerium treated group was improved in this assessment. Preliminary results showed that the mean IL2Rα level had dropped only to 73% of the first day levels (Table 6; *manuscript in preparation*). The *in vitro* IL2 production by burn survivor's lymphocytes, which had been consistently below normal levels (Figure 2) was found to be in the normal range (Figure 9; *manuscript in preparation*) in comparable patients bathed once in cerium nitrate (Table 7; *manuscript in preparation*). Thus, preliminary results suggested that this treatment attenuated the immunosuppressive activity. This was demonstrable even in burn patients whose burns may not necessarily have led them to mortality, and who thus may not have been distinguished by a survival/non-survival classification.

Further examination of such results is continuing but it is noteworthy that cerium treatment promises hope for severely burned patients, to survive against previously estimated enormous odds. Casualties of burns on the battlefield, if treated one way or another with cerium, may therefore look forward to a more enthusiastic triage, the optimism coming equally from the military medical services who bear this responsibility.

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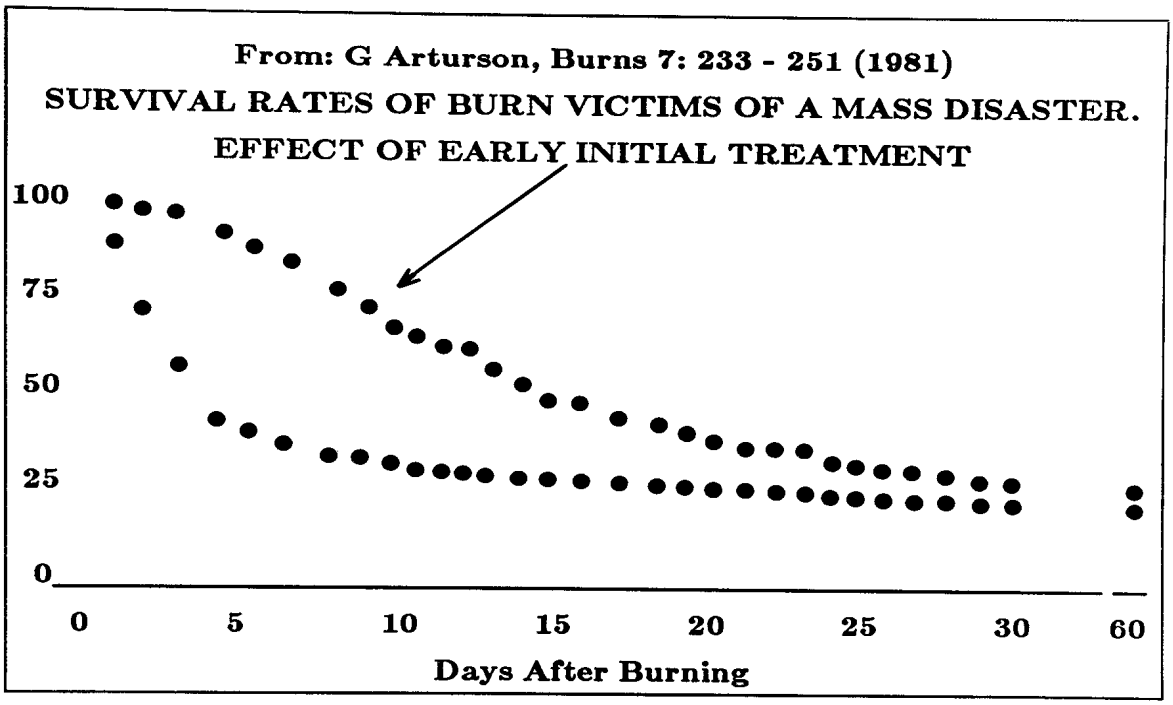

Figure 1. Percent survivors in each of two groups of burn victims, one (upper curve) which had received early fluid resuscitation (Ref. 3).

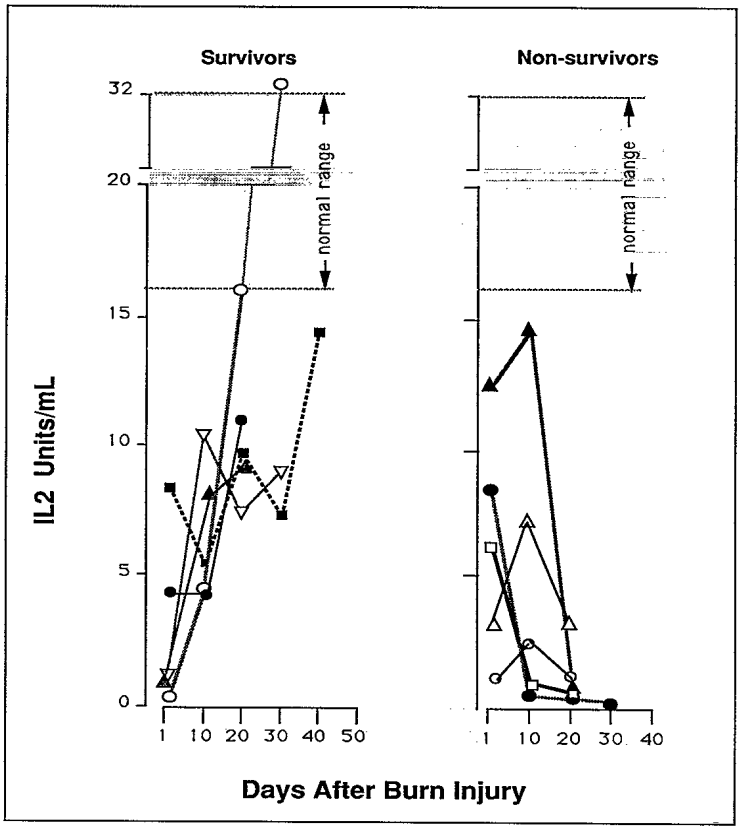

Figure 2. IL2 production by burn patients' PBMC stimulated by mitogenic lectin *in vitro* (Ref. 36).

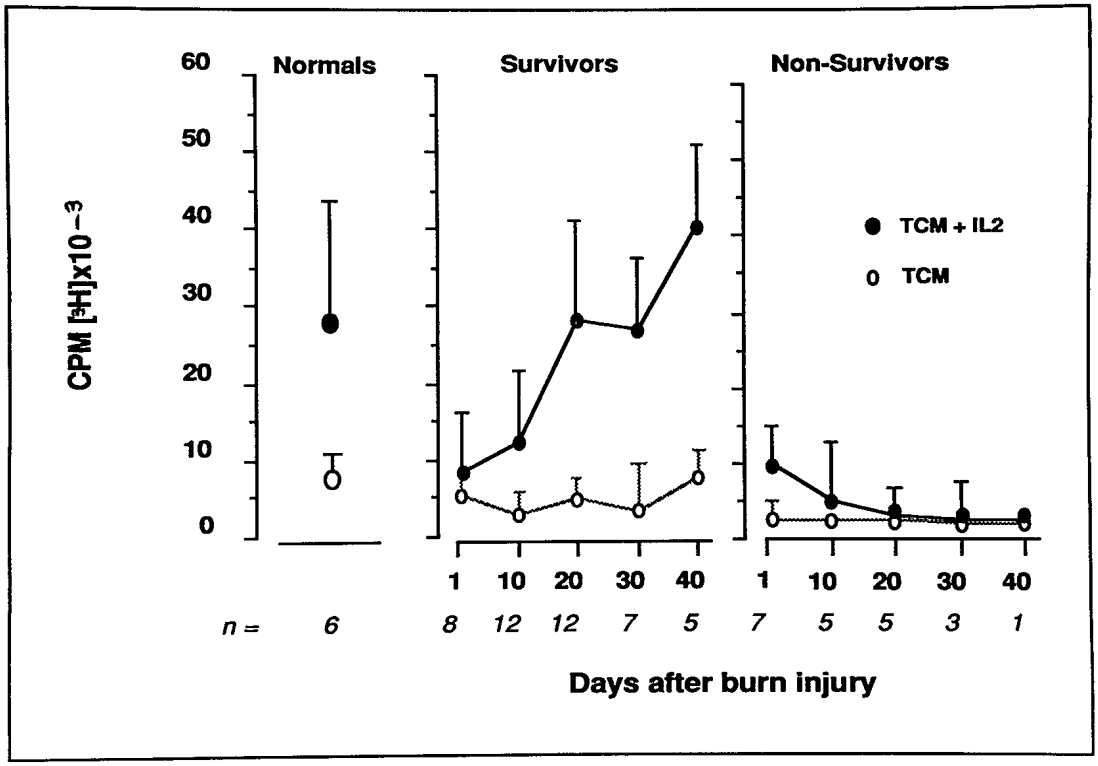

Figure 3. Mitogenic effect of exogenous IL2 added to the tissue culture medium (TCM) of lymphocytes from normal control subjects and burn patients distinguished as survivors or non-survivors (Ref. 37).

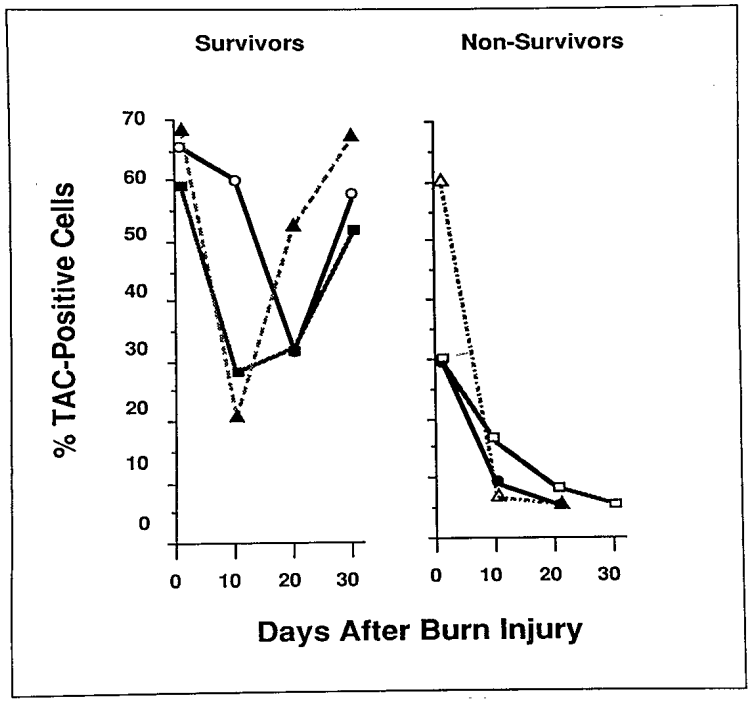

Figure 4. Percentage of the PBMC population displaying the surface IL2 receptor (IL2Rα, or TAC), after *in vitro* cultivation with mitogen (Ref. 36).

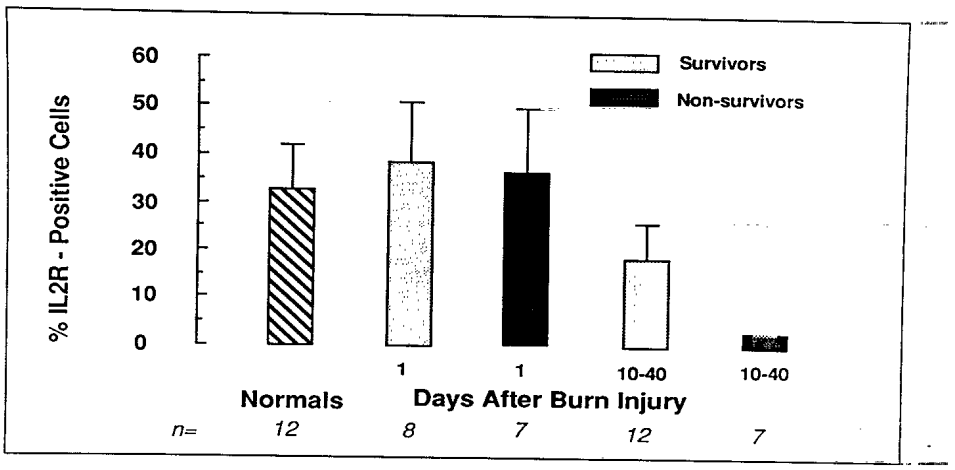

Figure 5. Percentage of the PBMC population displaying the IL2 receptor (IL2Rα or TAC) in normal subjects and in burn patients on Day 1 and at the time of the patients' lowest level of surface receptor (Ref. 37).

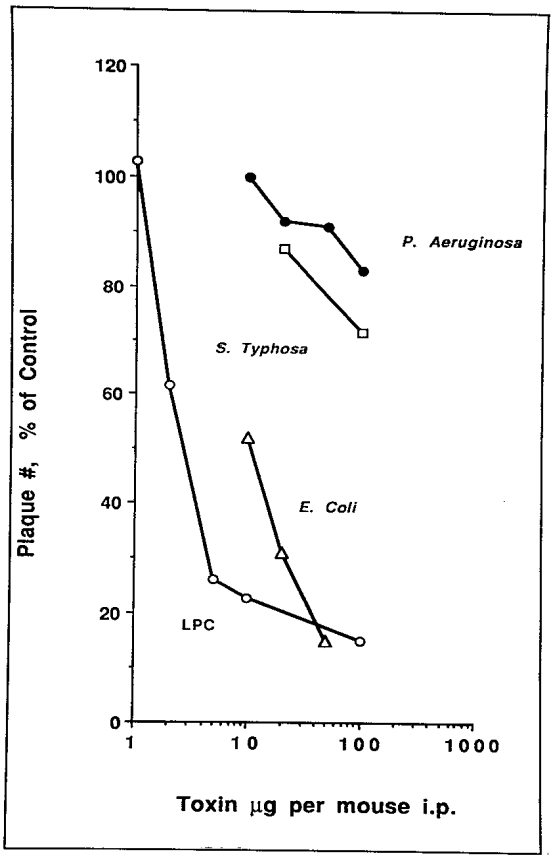

Figure 6. Effect of toxins on the plaque forming cell response *in vivo* in mice responding to antigen. Endotoxins from three bacteria, and the lipid protein complex (LPC) from burned skin were given two days before the antigen sheep erythrocytes (Ref. 34). For comparison on a molar basis weight of toxin should be divided by 3 million for LPC and by 20,000 for endotoxins.

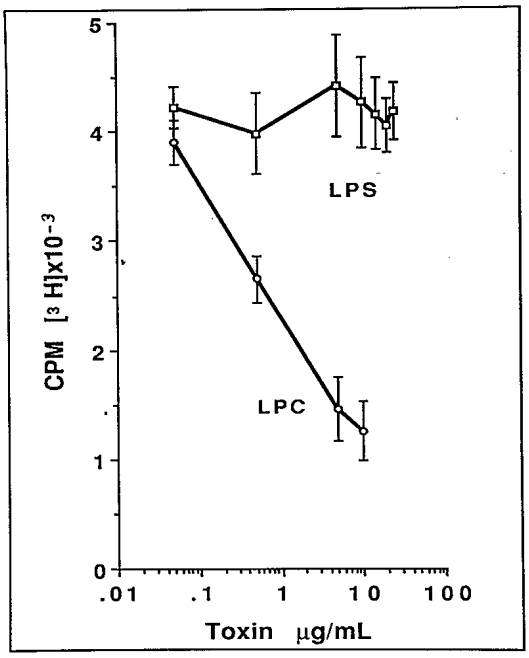

Figure 7. Effect of lipid protein complex (LPC) on growth of IL2-dependent cells in culture, in the presence of IL2, in comparison with the effect of endotoxin (lipopolysaccharide; LPS).

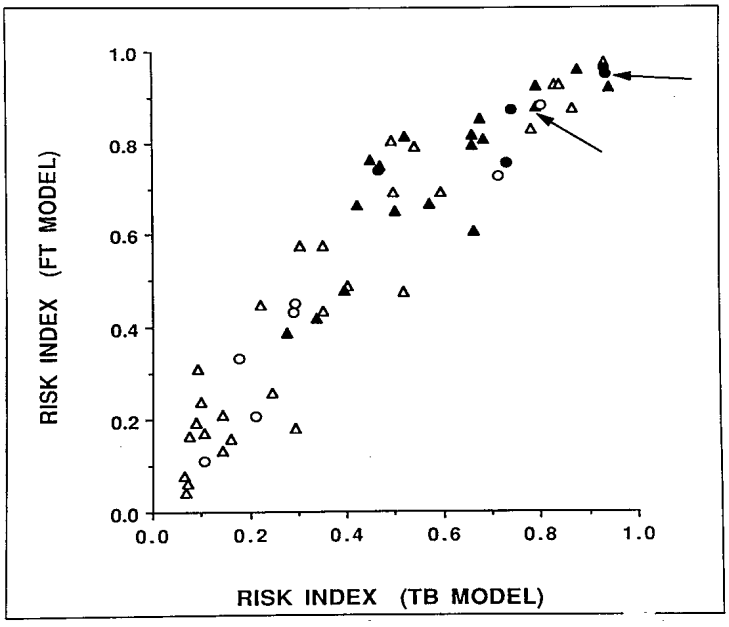

Figure 8. Relationship of two risk scores (Ref. 26) calculated on a population of burn patients given one bath of cerium nitrate. Risks were based on Full Thickness burns area (FT model) and on % TBSA (TB model) of burn patients. All patients survived except three. Two deaths are indicated by arrows (for one, a suicide, 90% TBSA, further resuscitation attempts were cancelled; the other suffered a pulmonary embolism. The third, with a fractured femur and internal injuries, had a TB Risk of 0.66 but no FT data was available.

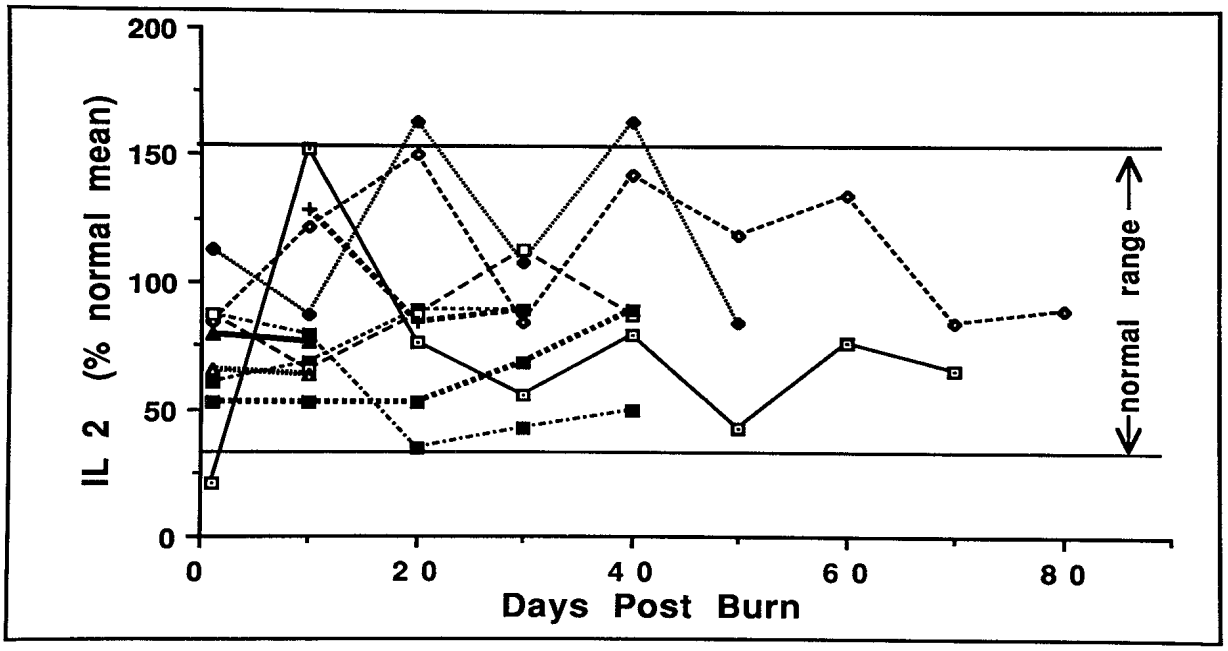

Figure 9. IL2 production by PBMC *in vitro*, taken from cerium treated (one bathing only) burn patients. Individual patient values are depicted as a percentage of the mean value of IL2 production by lymphocytes from normal subjects, assayed at the same time. The range of values found among normals is given as upper and lower limits.

Table 1. Percent of casualties of warfare suffering burns.

WARFARE BURN CASUALTY STATISTICS

Burn Victims as Percent of Casualties

WW II	(Brit)	1.5%
KOREA	(US)	2.8%
VIET NAM	(US)	4.6%
YOM KIPPUR	(Israeli)	10%
FALKLANDS	(Brit)	14%
LEBANON 82	(Israeli)	8.6%

Table 2. Observations that indicate that particularly T cell immune functions are impeded by burn injury.

THERMAL INJURY PRODUCES IMPAIRMENT OF CELLULAR IMMUNITY (T CELL IMMUNITY)

CIRCULATING SUPPRESSIVE FACTORS INHIBIT

1) T CELL CYTOLYTIC ACTIVITY
2) PROLIFERATIVE RESPONSE TO T CELL MITOGENS
3) PROLIFERATIVE RESPONSE TO ALLOGENIC CELLS
4) DELAYED HYPERSENSITIVITY
5) ALLOGRAFT REJECTION

Table 3. Peripheral blood components indicating that peripheral blood mononuclear cells (PBMC), the cells isolated for the *in vitro* immunology studies, make up only 27% of the leukocytes.

BLOOD COMPONENTS

PLASMA	**55% VOLUME**	**91% Water**
	8% PROTEIN	**0.9% SALTS**
FORMED ELEMENTS		**45% VOLUME**
	PLATELETS	**300,000 per μL**
	RED CELLS	**5 x 10^6 per μL**
	WBC (LEUKOCYTES)	**5 - 10 x 10^3 per μL**

GRANULOCYTES (POLYMORPHONUCLEAR CELLS)
73% of WBC

AGRANULOCYTES (MONONUCLEAR CELLS)
22% of WBC = LYMPHOCYTES
5% of WBC = MONOCYTES/MACROPHAGES

Tables 4 and 5. Mortality rate of patients treated over their course of hospitalization with topical cerium nitrate 0.04M solution applied to gauze dressings (From Refs. 17 and 18).

From Monafo et al. SURGERY **80:** *465 (1976)*

MORTALITY RATE OF PATIENTS TREATED WITH TOPICAL CERIUM NITRATE

SURFACE AREA BURNED (%)	N°. PATIENTS	N°. DEATHS PREDICTED	N°. DEATHS OBSERVED
1 - 19	32	1.1	0
20 - 39	16	3.3	1
40 - 96	12	8.7	6
Total	60	13.1	7

From Monafo et al. ARCH. SURGERY **113:** *397 (1978)*

MORTALITY RATE OF PATIENTS TREATED WITH TOPICAL CERIUM NITRATE

SURFACE AREA BURNED (%)	N°. PATIENTS	N°. DEATHS PREDICTED	N°. DEATHS OBSERVED
70 - 79	4	3	0
80 - 89	5	5	2
90 - 100	7	7	6
Total	16	15	8

Table 6. Percent of PBMC expressing surface IL2 receptor in vitro, comparing cells from burn patients treated in the standard fashion and those treated by one cerium bath. Lowest mean IL2R level is higher in cerium patients although due to large variance is not significantly increased.

PERCENTAGE OF CELL POPULATION EXPRESSING IL2R

n	*AGE*	*TBSA*	*3°*	*% IL2R+ DAY 1*	*LOWEST % IL2R+ PB PERIOD*
STANDARD TREAMENT					
SURVIVORS					
8	**31.0 ± 8.2**	**42.0 ± 12.7**	**30.8 ± 13.9**	**38.3 ± 11.7 (26 - 51)**	**18.4 ± 7.3 (48%) (7 - 30)**
NON-SURVIVORS					
7	**54.6 ± 16.4**	**60.7 ± 22.0**	**46.7 ± 14.3**	**36.6 ± 16.4 (17 - 62)**	**<5 (0 - 6)**
CERIUM BATHED					
10	**37.2 ± 12.4**	**35.0 ± 9.3**	**27.8 ± 13.3**	**32.3 ± 21.8 (4 - 63)**	**23.7 ± 15.2 (73%) (7 - 58)**
NORMALS					
10	**36.8 ± 8.2**				**36.2 ± 9.7 (17 - 51)**

Table 7. IL2 production in vitro by lymphocytes taken from patients given standard treatment and those given one cerium bath. Values for each patient sampling over his hospitalization period were averaged and the mean of these averages is given. There is no significant difference between normals and cerium treated patients.

IL2 PRODUCTION BY LYMPHOCYTES IN VITRO

n	*AGE*	*TBSA*	*3°*	*IL2 U/ml*	
STANDARD TREATMENT					
SURVIVORS					
6	**38.0 ± 7.6**	**35.3 ± 8.7**	**27.0 ± 11.7**	**9.1 ± 2.9 (0 - 32)**	
NON-SURVIVORS					
7	**53.7 ± 12.1**	**57.0 ± 13.6**	**49.0 ± 20.4.**	**5.0 ± 3.7 (0 - 22)**	**p < 0.001**
CERIUM BATHED					
10	**37.2 ± 12.4**	**35.0 ± 9.3**	**27.8 ± 13.3**	**31.6 ± 7.6 (8 - 62)**	
NORMALS					**n.s.**
10	**36.8 ± 8.2**			**37.5 ± 17.2 (16 - 60)**	