Kinetics of Humoral Responsiveness in Severe Thermal Injury

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Severe thermal injury has the capacity to increase the rate of generation of antibody-forming cells in mice. The intensity of stimulation appears to be proportional to the extent of injury. The effect has been observed in animals burned within 1 hr before or after sensitization with test antigen(s), and persists up to 14 days after injury. Thereafter, the stimulatory effect wanes, and disappears by the 21st day after burning. Responses to T-cell (thymus derived lymphocytes) dependent antigens (sheep erythrocytes; sheep erythrocytes coupled to TNP) and to antigens not requiring T and B-cell (bone marrow derived lymphocytes) cooperation (DNP-Ficoll) appear to be equally affected by thermal injury. The mechanisms underlying this form of enhanced antibody response are not clear. The data, however, support the possibility that the burn wound may release factor(s) capable of enhancing humoral responsiveness in the injured animal. Such factor(s) do not appear to be endotoxins.

A STEADILY GROWING ARRAY of experimental and clinical data suggests that thermal injury is associated with significant alterations in immunological responsiveness, and that a better understanding of such changes may contribute to improvements in the morbidity and mortality of severe burns. A number of reports[22,37,46] have provided early evidence of decreased reactivity to skin allografts in burned patients; studies in this laboratory[75] yielded similar results in burned animals, and Alexander and Moncrief[2] have confirmed and extended these data in human subjects. A variety of other alterations in cellular immune mechanisms has been noted in thermal injury. Burned animals[76,95] and human subjects[17,70] have been shown to lose the capacity to exhibit skin hypersensitivity reactions of the delayed type, with a direct relationship between the extent of injury and the degree of inhibition of skin reactivity.[18] Thermal injury has also been associated with decreases in the output of thoracic duct lymphocytes[18,77] and with a depletion of mononuclear cell elements in the skin of burned patients, as studied by the Rebuck chamber technique.[60] The depressed levels of reticuloendothelial system function observed in severe thermal injury,[21,31,34,78–80,83,87,94] and recent evidence that thermal injury may trigger immunologically mediated processes leading to organ-specific damage[1,10,11,16,20,25,43,56,61,74,81,90] have added new dimensions to the study and management of severe burns.

Overwhelming sepsis, usually as a consequence of infection by normally non-pathogenic microorganisms remains the most lethal threat to the survival of the severely burned patient today.[73,93] Jackson, Lowbury and Topley[38] and Liedberg and his associates[52] pointed to septicemia as a common cause of death in burns, and highlighted the role of Group A hemolytic streptococci in the failure of skin allografts.[53] Many authors[3,6,9,23,35,47,–57,67,89,92] then noted a shift to Gram-negative bacteria as the predominant cause of septicemia a few years later, and Lindberg, Moncrief and Mason[55] showed a direct relationship between the surface area of the burn and susceptibility to infection. Liedberg[54] has also noted that thermal injury enhances the invasiveness and lethality of intraperitoneally injected *Pseudomonas aeruginosa* in guinea pigs, and increased susceptibility to *Pseudomonas* septicemia has been described in burned rats by McRipley and Garrison[63] and in burned mice by Markley et al.,[58] Millican, Evans and Markley[65] and Rosenthal[84].

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The observation of Langohr, Owen and Cope,[51] Fox and Lowbury[29] and of Jones and Lowbury[41] that burned subjects appear to retain the ability to form serum antibodies against the bacteria colonizing their wounds has stimulated interest in the possible use of active and/or passive methods of immunization in order to increase antibody formation and to protect the injured host from septicemia. Jones, Jackson and Lowbury[42] and Millican, Evans and Markley[65] have provided evidence of the potential usefulness of this approach in mice, and there have been some encouraging exploratory studies in human subjects.[4,27,28,86] Further progress in this important area has been hindered, however, by the relative paucity of experimental data available on the capacity of the burned host's antibody-forming cells (T- or thymus derived and B- or bone marrow derived cells) to muster and sustain specific humoral responses after challenge with antigen(s) of different molecular configuration. The present study is aimed at an assessment of the ability of burned animals to initiate and maintain antibody responses against T-cell-dependent antigens, using sheep erythrocytes (sRBC) and sRBC substituted with trinitrophenyl (sRBC-TNP) as the prototype,[12,19,45] and against T-cell independent, B-cell dependent antigens, using Ficoll (F) coupled with Nε-dinitrophenyl-L-lysine (DNP) as the test material.[62,69] The results are consistent with the conclusion that experimental thermal injury may be associated with significant increases in the rate of proliferation of antibody-forming cells in the burned host.

Materials and Methods

Experimental Animals and Method of Thermal Injury

Male Swiss-Webster and $C_{57}Bl_6$ mice weighing 25–35 gm and maintained on a standard Purina pellet diet were used. On the day before burning, all mice were weighed and hair was removed from the groin to the axilla with electrical clippers. The body surface of each animal was calculated by the method of Benedict,[14] and the mice were anesthetized with ether prior to contact of a predetermined skin surface area with a thermally regulated metal surface maintained at 200°C. Use of asbestos frames of known size, with metronome-controlled exposure time (2 sec for the back and flanks and 1 sec for the abdomen) and constant pressure produced full-thickness skin burns involving 5%, 10%, 15%, 20%, 30%, and 40% of the body surface area (BSA). The depth of the inflicted burns was confirmed routinely by dissection and histological examination. Immediately after injury, 10 ml of isotonic saline solution per 100 gm of body weight were given intraperitoneally, and the animals were housed in a controlled environmental chamber (Modulab, Lab-Line Instruments, Inc.) maintained at 27 C ± 1 C and 40% humidity; water was provided ad libitum. The burned mice were observed for 21 days after injury, and the mortality was recorded daily. The results observed in the first pilot studies of 215 $C_{57}Bl_6$ and 521 Swiss-Webster mice provided the baseline for determination of the burns mortality by this technique. There was a mortality of 5.2%, 9.8%, 10%, 24%, 58%, and 86% at 21 days for full-thickness burns involving a BSA of 5%, 10%, 15%, 20%, 30%, and 40%, respectively. In a search for a compromise between the aim at a burn of maximum severity and the survival of sufficient numbers of animals to permit the planned studies, groups of mice selected for sensitization with sRBC, sRBC-TNP, of DNP-F were subjected to 15% or 30% BSA injuries.

Antigenic Preparations

Sheep (s) and horse (h) red blood cells (RBC) (Animal Blood Center, Syracuse, N.Y.) were washed three times in balanced salt solution (BSS) and were used as antigens.[66] The washed cell suspensions were adjusted to the required concentration in standard fashion.[30] Erythrocytes were lightly and heavily substituted with TNP (sRBC-TNP and hRBC-TNP) by the methods of Rittenbury and Pratt[82] and of Kettman and Dutton,[48] respectively. RBC's were substituted with DNP by the technique of Trump.[91]

In the preparation of DNP-Ficoll,[62] 1.9 gm of the sucrose polymer Ficoll (Pharmacia Fine Chemicals, lot 2,300, mol. wt. 400,000 d) were suspended in 4.0 ml of distilled water, and 4.0 ml N NaOH with 0.5 gm $KHCO_3$ were added. Upon full solution, a freshly prepared suspension of 200–600 mg cyanuric chloride in 2 ml dimethyl formamide was added, and the mixture was stirred at room temperature for 2–4 min. One gm of Nε-dinitrophenyl-L-lysine (DNP) in 1.0 ml distilled water at pH 9.0 was then added. The mixture was stirred overnight and was dialyzed for 2 weeks against distilled water. It was then dialyzed for 3 days against isotonic saline solution. The preparation was centrifuged at 2,000 g and the supernatant was passed through a 0.22 μ pore-sized filter. The concentration of DNP in the final preparation was determined spectrophotometrically at 365 nm, using a molecular extinction coefficient of 16,400. The carbohydrate content was determined by the phenol sulfuric acid test.[24]

Method of Tissue Culture

Mouse spleen cell cultures were initiated and maintained by the technique of Mishell and Dutton,[90] with addition of 10^{-5} M 2-mercaptoethanol to the culture medium as recommended by Glick.[32] Generally, 1.5 to 2×10^7 spleen cells in each culture were challenged with 4×10^6 unmodified RBC, or with fresh RBC heavily

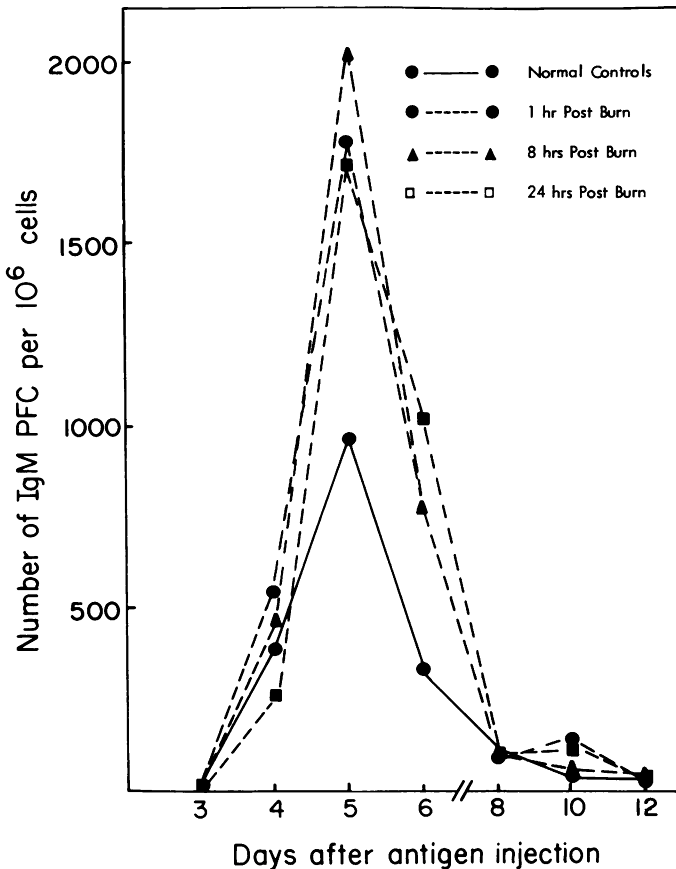

FIG. 1. Number of IgM PFC in the spleen of mice injected with 2×10^8 sRBC at various time intervals after 30% skin burn.

substituted with TNP. The generation of IgM-producing cells was determined by counting the number of direct plaque-forming cells (PFC) in each culture, by the plaque technique of Jerne, Nordin and Henry,[40] as modified by Mishell and Dutton.[66] In those experiments where anti-TNP responses were measured, lightly substituted RBC-TNP were used for plaquing.

Guinea pig serum was absorbed three times with the appropriate RBC, and served as the source of complement in the plaquing technique. The number of PFC counted on each day reported represents the average of counts performed in three simultaneously prepared culture dishes, which were plaqued in triplicate against each RBC preparation tested. Since earlier studies have shown that the number of PFC formed in response to DNP-RBC or to TNP-RBC is regularly the same,[12] and TNP-RBC preparations are more stable than DNP-RBC, the former were generally used in plaque assays during the present study.

Basic Experimental Design

1. Groups of 200 $C_{57}Bl_6$ mice were first subjected to a 15% or 30% BSA standard burn. Each mouse was then injected intravenously with 2×10^8 sRBC at 1, 8, or 24 hrs, or at 7 or 14 days after injury. Beginning with the third day after the injection of sRBC, 5 to 10 animals were sacrificed daily. Spleen cell cultures were prepared from each mouse, and the cells were plaqued against sRBC; a concurrent number of control unburned mice was treated in the same manner.

2. The same protocol and number of animals were used in experiments with mice which were sensitized intravenously with lower doses of sRBC (2×10^7 cells), with sRBC-TNP (2×10^8 cells), or with a T-cell-independent antigen, DNP_{32}-Ficoll (20 μg). When the IgM PFC responses to TNP or DNP were measured, hRBC-TNP were used in the plaquing technique.

3. Additional groups of 200 mice each were first injected intravenously with 2×10^8 s RBC, 2×10^7 sRBC, 2×10^8 sRBC-TNP, or with 20 μg DNP_{32}-F, and were then exposed to a 15% or a 30% BSA burn within 1, 8, or 24 hr after the injection of antigen. The IgM PFC responses observed in each group of animals were determined in the same manner as described above. The results were compared with those obtained in concurrent unburned control mice.

The standard Student's Test was used for statistical analysis of the results, with $P \leq 0.01$ accepted as the level of significance of differences observed between

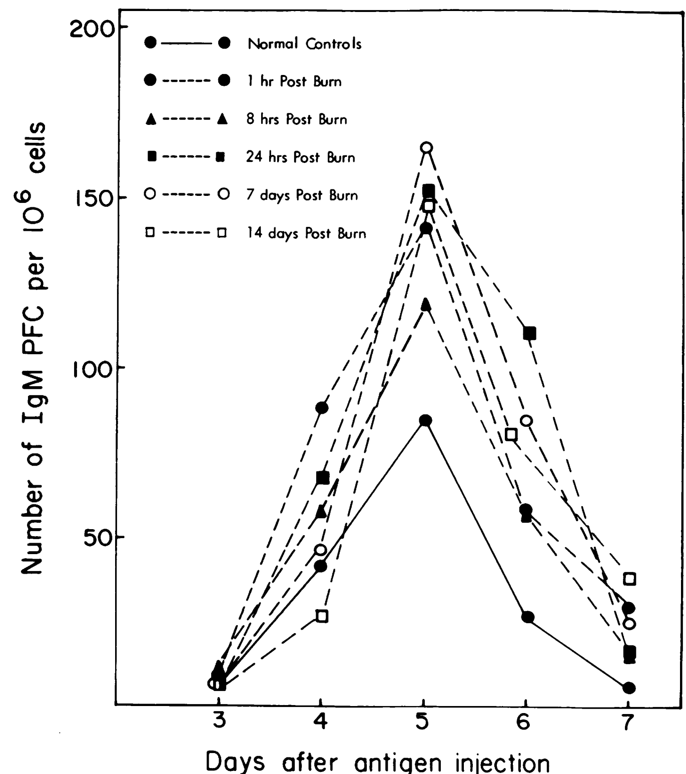

FIG. 2. Number of IgM PFC against TNP in the spleen of mice injected with 2×10^8 sRBC-TNP at various time intervals after 30% skin burn.

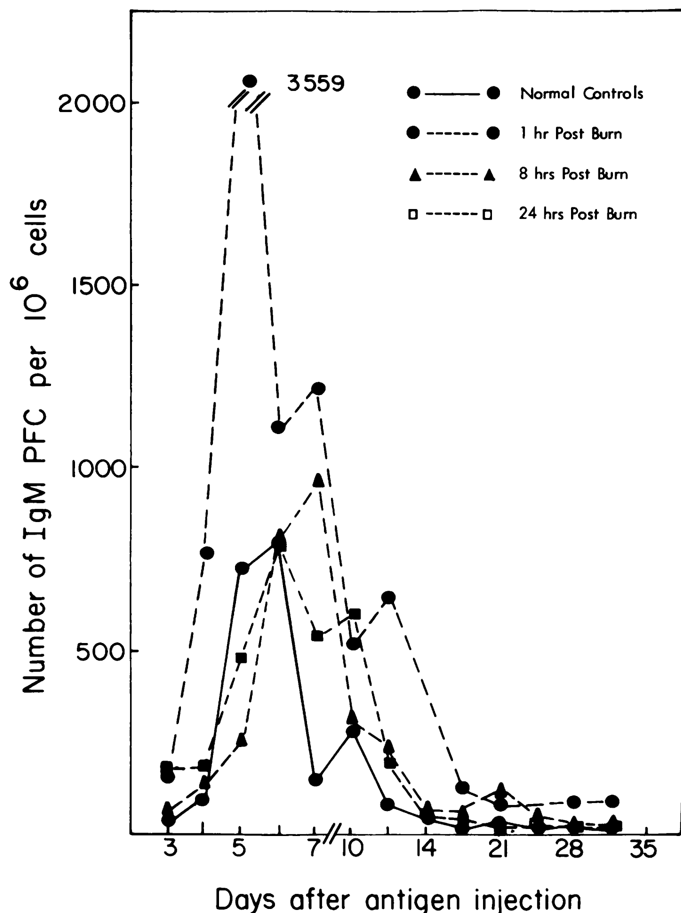

FIG. 3. Number of IgM PFC against TNP in the spleen of mice injected with 20 μg DNP_{32}-F at various time intervals after 30% skin burn.

cell cultures obtained from burned and from uninjured concurrent control mice.

Results

Antibody Formation in Mice Sensitzed after Thermal Injury

In the first group of experiments, mice received a low dose of sRBC (2×10^7 cells) at 1, 8, or 24 hrs, or at 7 or 14 days after a 30% BSA burn, and the rate of generation of IgM SRBC plaque forming cells (PFC) in the spleens of such animals was compared sequentially for 10 days with the results obtained in unburned mice. Equal total numbers of PFC were produced in burned and in normal mice. The peak period of generation of PFC was on the 7th day in burned mice, and on the 6th day in control animals. This 24-hr lag in the maximum rate of PFC generation occurred only in mice given low doses of sRBC at 1, 8 or 24 hrs after burning. It was not detectable in mice injected with sRBC at 7 or 14 days after thermal injury.

Somewhat different results occurred in mice given the optimal sensitizing dose of 2×10^8 sRBC.[12] As noted in Fig. 1, there were significant augmentations in the numbers of sRBC PFC formed in mice given sRBC at 1, 8 or 24 hrs after a 30% BSA burn when compared to the results in control mice ($P < 0.01$ in each instance). An increased generation of PFC was still detectable, but less pronounced, in mice given sRBC at 7 and 14 days after burning (P = 0.01). Fig. 2 illustrates similar findings in mice injected with sRBC-TNP at various intervals after injury and tested for IgM responses against hapten alone. There was a marked increase in the generation of PFC against TNP, and such enhanced responses occurred in mice sensitized within 1, 8, or 24 hr, or at 7 or 14 days after injury, when compared to normal mice ($P < 0.01$ in each case); the responses returned to normal levels at 21 days after burning. Roughly parallel data were obtained in mice sensitized in the same manner after exposure to 15% BSA burns. The levels of increased generation of IgM PFC were much lower, however, than the results observed after a 30% BSA injury.

Fig. 3 outlines the results of sensitization of mice with a T-independent antigen, DNP_{32}-F, injected within 1, 8

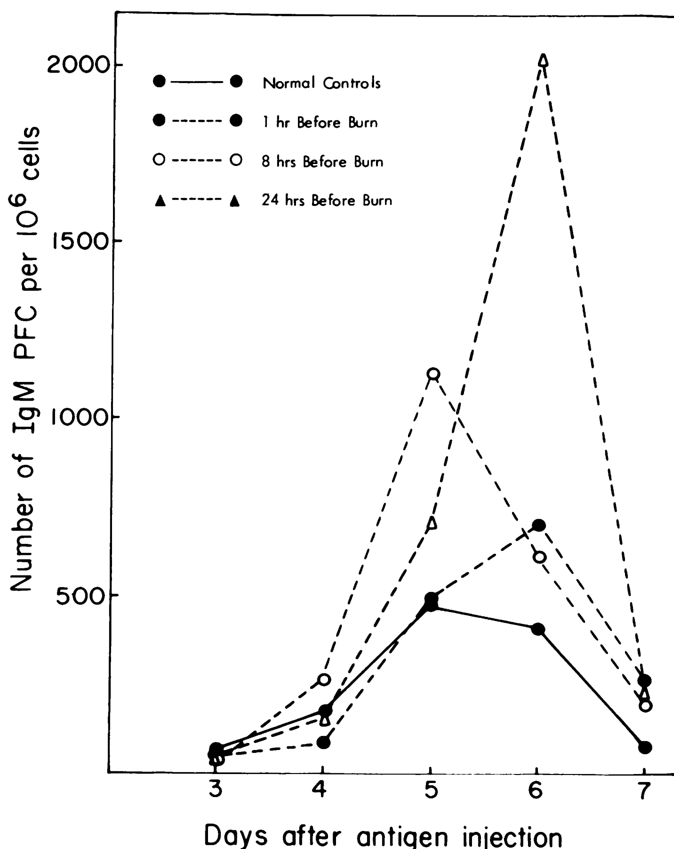

FIG. 4. Number of IgM PFC in the spleen of mice injected with 2×10^8 sRBC at various time intervals before 30% skin burn.

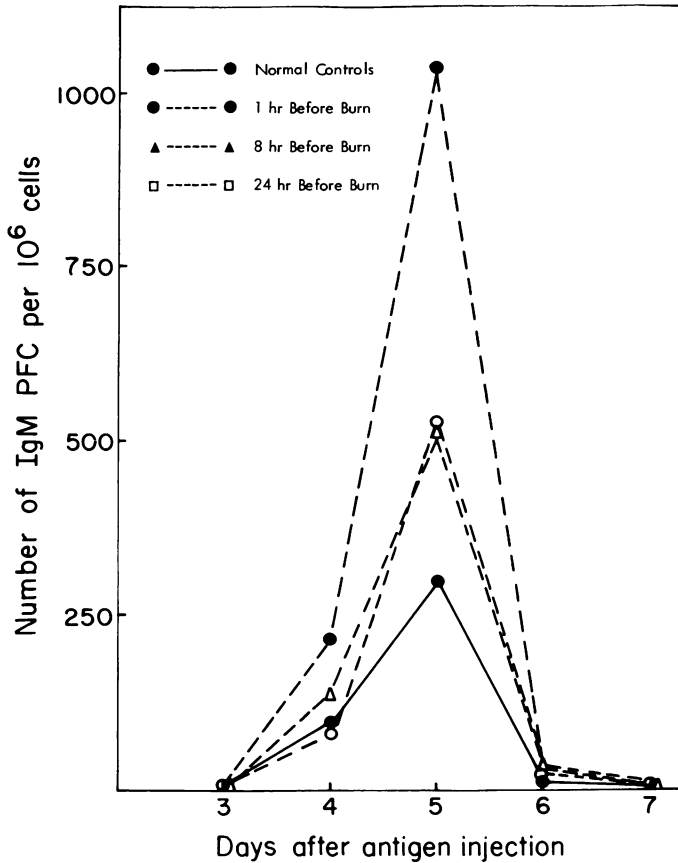

FIG. 5. Number of IgM PFC against TNP in the spleen of mice injected with 2×20^8 sRBC-TNP at various time intervals before 30% skin burn.

or 24 hrs or at 7 days after a 30% BSA burn. The number of anti-hapten IgM PFC was markedly increased in animals given DNP 32-F at 1 hr after injury, when compared to control results ($P < 0.01$). There was only a slight (not statistically significant) increase in the generation of PFC in mice sensitized at 8 hr after burning; mice given DNP_{32}-F at 24 hr or 7 days after injury produced the same number of PFC as the unburned control animals.

Antibody Formation in Mice Sensitized Before Thermal Injury

Groups of mice sensitized with 2×10^8 sRBC were exposed to a 30% BSA burn within 1, 8 or 24 hr after the injection of antigen(s). As illustrated in Fig. 4, there was a clear-cut and progressive enhancement in the generation of IgM sRBC PFC as the time interval between the injection of antigen(s) and of burning was increased. The most marked effect occurred in mice exposed to thermal injury within 24 hr after sensitization ($P < 0.01$, when compared to control mice). The number of PFC generated by mice in this group reached a peak which was 4 times greater than the PFC generated in unburned control mice. Fig. 5 illustrates the results obtained with 2×10^8 sRBC-TNP were used; there were also significant increases in anti-TNP IgM PFC ($P \leq 0.01$). The most pronounced enhancements in response occurred, however, in animals exposed to thermal injury at 1 hr after sensitization with sRBC-TNP.

Fig. 6 highlights the effects of sensitization with DNP_{32}-F in mice subjected to 30% BSA burns within 1, 8 or 24 hrs after injection of this T-cell-independent antigen. There were augmented anti-TNP IgM PFC responses in mice burned at 1 or 24 hr after sensitization ($P \leq 0.01$). The rate of generation of IgM PFC against TNP in mice burned at 8 hr after sensitization was, however, roughly comparable to the results observed in unburned animals.

Effects of Endotoxin and of Thermal Injury Upon the Generation of IgM Antibody-Forming Cells

Gram-negative endotoxins have the capacity to markedly enhance the immune response. A possible explanation for the enhanced antibody responses observed in burned animals might therefore have been the release of endotoxin or of endotoxin-like materials at the site of injury. To study this possibility 200 normal mice were injected intravenously with 100 μg of *E. coli* lipopolysaccharide (Difco Labs), and another group of 200 mice was given 30% BSA burns. Spleen cell cultures were prepared at 2, 3, 4 and 5 days after treatment, and the

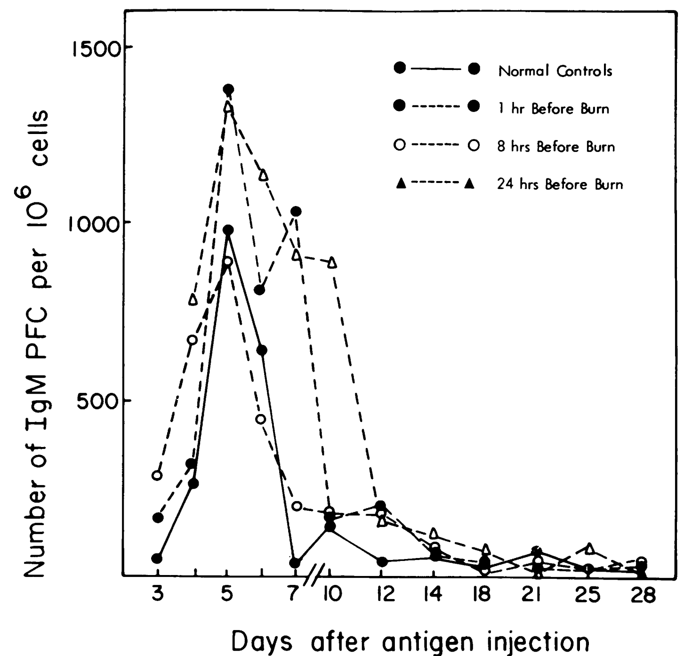

FIG. 6. Number of IgM PFC against TNP in the spleen of mice injected with 20 μg DNP_{32}-F at various time intervals before 30% skin burn.

TABLE 1. *Generation of Spleen IgM PFC of Mice Injected With LPS or After 30% Skin Burn*

	Number of PFC per 10^6 Cells at Day:							
	Day 2		Day 3		Day 4		Day 5	
Mice Treated	sRBC	hRBC-TNP	sRBC	hRBC-TNP	sRBC	hRBC-TNP	sRBC	hRBC-TNP
100 μgr LPS	51 ± 5.0	48 ± 8.0	123 ± 11.0	154 ± 18.0	111 ± 9.0	115 ± 15.0	38 ± 4.0	28 ± 6.0
30% Burn	1 ± 0.5	0	2 ± 1.0	1 ± 0.5	1 ± 0.5	2 ± 1.0	1 ± 0.5	0

rate of generation of IgM PFC against sRBC and hRBC-TNP was compared in the two groups. As shown in Table 1, mice injected with endotoxin produced significant numbers of IgM PFC against sRBC and hRBC-TNP. In contrast, animals subjected to 30% BSA burns failed to generate IgM PFC in excess of background levels. This result provides indirect support to the possibility that thermal injury may not cause enhanced humoral responses as a consequence of the release of endotoxin.

Discussion

It is generally agreed that thermal injury is associated with a wide variety of serological alterations. Arturson et al.,[7] Kohn and Cort,[49] and Munster, Hoagland and Pruitt[71] have noted decreases in immunoglobulin levels in the first 48 hr after injury, in parallel with falls in total serum protein concentrations. The IgG and IgA components appeared to be the most severely affected, with relatively little change in IgM—possibly because IgM molecules do not migrate freely through the vascular wall. The early falls in immunoglobulin levels were followed by a gradual return to normal or elevated values. Arturson and Fjellstrom[8] have documented falls in all factors of complement, with correction of the defect within 7 to 10 days, and Kohn[50] and Goldberg and Whitehouse[33] have detected F(ab')2-like fragments of immunoglobulin similar to those produced by in vitro papain digestion in the serum of severely burned patients. Attempts to relate these changes to the ability of burned subjects to initiate and sustain humoral immunological responses have, however, yielded contradictory results. Balch[13] observed a normal capacity to form serum antibodies in burned patients, and Markley, Smallman and Evans[58] have described normal humoral responses to sheep erythrocytes in burned mice. On the other hand, Alexander and Moncrief[5] have noted diminutions in the ability of burned patients and experimental animals to respond to primary antigenic challenge, although there was no apparent interference with secondary humoral responses in patients given tetanus toxoid booster immunizations. In an extension of these studies, Alexander and Moncrief[2] assessed the response of burned rats to a variety of antigens. These authors reported normal serum antibody production to soluble extracts of *Pseudomonas aeruginosa* and to heat-killed *P. aeruginosa*, slightly increased responses to *S. typhosa* somatic antigen, and clear-cur diminutions in the response of burned rats to heterologous (human) erythrocytes.[2] Messerschmidt, Langendorff and Bomer[64] obtained similar results in burned and in irradiated mice, although the initial inhibition of hemagglutinin responses to heterologous erythrocytes seen in these animals was followed by an apparent rise in antibody titer in the second week after injury. In contrast, Mortensen and Eurenius[68] have recently reported increased antibody responses to sheep erythrocytes in burned rats.

The results of the present study indicate that thermal injury is associated with significant enhancements in the rate of generation of antibody-forming cells in response to T-cell dependent antigens (sRBC, sRBC-TNP) in mice. The effect is most pronounced in mice immunized within 24 hrs after burning; it persists for 14 days and is no longer detectable at 21 days. The effect appears to be roughly proportional to the extent of injury, in that it is less pronounced in mice subjected to 15% rather than 30% BSA burns. When the sensitizing dose of sRBC is decreased to 2×10^7 cells, the rate of production of PFC is not affected, and a 24-hr delay occurs in the peak period of PFC generation, when compared to normal mice. The result points to the importance of antigen dosage as a potential variable in determining the outcome of this type of experiment.

Severe burns have been shown in similar fashion to increase the rate of generation of IgG PFC against a T-cell-independent antigen, DNP_{32}-F. In contrast to the enhancements of PFC generation noted in response to sRBC or sRBC-TNP, however, this effect was only significant in animals given DNP_{32}-F within 1 hr after injury. The results observed in mice injected with the same dose of DNP_{32}-F at 8 hrs, 24 hrs, or 7 days after injury were comparable with those obtained in control animals. The reasons for this difference are not clear at present, beyond the possibility that humoral response(s) to DNP_{32}-F, as a T-cell-independent, B-cell stimulator, may be mediated by different cellular pathways than T-cell-dependent antigens (sRBC, sRBC-TNP), and that the cell population(s) involved vary in their susceptibility to the effects of thermal injury.

In further studies of the enhancement of humoral responses by thermal injury, mice sensitized with the same antigens underwent 30% BSA burns at various intervals after immunization. The rate of generation of IgM PFC in mice injected with sRBC increased progressively as the interval between the time of sensitization and of injury increased from 1 hr to 24 hr; maximum stimulation of PFC production occurred in the 24 hr group. There were similar increases in PFC generation in mice sensitized with another T-cell-dependent antigen, sRBC-TNP. In this instance, however, maximum stimulation occurred in mice burned within 1 hr after sensitization, with much less pronounced effects in animals burned at 8 or 24 hr after sensitization. The greater susceptibility of sRBC-TNP to hemolysis and in vivo degradation may account for this difference. When mice were injected with DNP_{32}-F, there were augmented anti-TNP IgM PFC responses in animals burned at 1 hr and at 24 hr after sensitization. Taken together, these data are consistent with the interpretation that thermal injury can stimulate significant increases in the rate of generation of antibody-forming cells in mice. This result is in agreement with the findings of Mortensen and Eurenius[68] in rats.

A number of possible explanations may be invoked for these results,[68] including: A) increased release of B-cells from bone marrow rendered hyperplastic by thermal injury; B) adjuvant effects of the burn wound, either as a consequence of the release of oligonucleotides from destroyed cells, or because of the absorption of endotoxins from Gram-negative microorganisms in the burn wound; C) changes in reticuloendothelial system (RES) function after thermal injury. The early effects of thermal injury militate against a role of bone marrow hyperplasia, and the fact that the level of stimulation of PFC decreases as the time interval after thermal injury increases is not consonant with an endotoxin adjuvant effect. Other studies which do not support a contributory role by endotoxin(s) are presented in this report.

The suggestion that the RES may be implicated in these enhanced humoral responses is in agreement with reports that the immunogenic activity of heterologous erythrocytes is normally destroyed by the liver and spleen.[30] Significant depressions in RES function occur during the early post-burn phase (8 to 24 hr), with gradual return to normal levels of activity by 7 days and somewhat increased levels of function in the next 2 to 3 weeks.[80] The lowered RES function noted in the first hours after injury may cause delayed degradation of immunogens in the burned animals' spleens and livers, with a resulting higher antibody response. This explanation is only valid, however, in those instances where antigen(s) is injected within 24 hr after injury. It is not applicable to the increased rates of PFC generation observed in mice sensitized at 7 or 14 days after burning, when the injured host's level of RES function has returned to normal or elevated levels. The latter enhanced humoral responses may, however, be related to a compensatory increase in the production of T-cells, following the early post-burn phase of T-lymphocyte depletion.[18,60,77]

The precise mechanisms whereby thermal injury appears to stimulate increased generation of antibody-forming cells are not clear at present; they appear, however, to be linked with the acute burn wound. This consideration raises the possibility that such enhanced humoral responses may be related to the release from the site of injury of stimulatory factor(s) other than endotoxin(s). The relationship between the extent of body surface area burned and the stimulatory effect of the resulting wound is in keeping with this possibility. The studies of Braun and Firshein[15] on the immunological adjuvant effects of oligonucleotides released from lysed cells may be of relevance to further studies of this possible biological effect of the burn wound. A number of conflicting studies have been reported regarding the altered antigenicity[26,36,44,72] and/or toxicity[39,85,88] of burned skin. There has been a paucity of data, however, on possible stimulatory effects of normal and/or altered tissue components of the burn wound upon the host's cellular or humoral state of immunological responsiveness. The results of this study point to the potential relevance of this approach to further studies of the pathophysiology of thermal injury.

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