Molecular mechanisms of decreased interleukin-2 production after thermal injury

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Background. *Among the fundamental immunologic abnormalities induced by serious traumatic or thermal injury are alterations in T cell activation, reduced lymphocyte interleukin-2 (IL-2) production, and associated depression of T lymphocyte proliferation. This study attempts to localize the cellular mechanisms underlying abnormal IL-2 production in thermal injury.*

Methods. *Following National Institutes of Health guidelines, 150 A/J mice were anesthetized, subjected to a 20% full-thickness scald burn injury or sham burn, and killed at intervals from 4 to 21 days later; splenocytes were harvested for in vitro studies. For measurement of IL-2 production, cells were cultured with either concanavalin A or a combination of the phorbol ester PMA, which directly activates protein kinase C, and the calcium ionophore A23187, which increases intracellular calcium. Cytokine mRNA expression was measured by Northern blot analysis and IL-2 production by bioassay.*

Results. *Both IL-2 production and IL-2 mRNA expression were consistently suppressed in concanavalin A–stimulated cells from burned mice compared with sham burns. This suppression of IL-2 and IL-2 mRNA also occurred when T cells were activated with PMA and A23187, bypassing the earlier stages of the signal transduction mechanism. IL-1β and tumor necrosis factor-α mRNA expression were consistently increased in burned animals, indicating that decreased IL-2 mRNA expression was specific to IL-2 and not representative of a global decrease in cytokine mRNA expression.*

Conclusions. *These results suggest that the principal cellular abnormalities that result in altered T cell activation and IL-2 production after thermal injury lie downstream of the initiating signal transduction events and before IL-2 gene transcription. (SURGERY 1993;114:407-15.)*

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IN SPITE OF MODERN THERAPEUTIC advances, sepsis remains a significant cause of death after burn injury.[1] Severe thermal or traumatic injury leads to depression of cell mediated immunity,[2] with abnormalities of T lymphocyte activation and proliferation in response to antigenic or mitogenic stimuli.[3] Our laboratory has previously shown that one of the major immunologic abnormalities in this setting is decreased production of interleukin-2 (IL-2) in response to mitogenic stimulation.[4, 5] IL-2 plays a central role in immunoregulation, supporting lymphocyte proliferation, promoting helper cell activity, and augmenting cytotoxicity.

When resting (G_0 phase) T lymphocytes are stimulated with mitogenic lectins such as concanavalin A (Con A), cells enter the G_1 phase of the cell cycle where they produce IL-2 and express IL-2 receptor proteins, thus becoming committed to activation. The earliest events in T cell activation from the stimulation of T cell receptors to IL-2 gene transcription have been the subject of intense investigation in recent years. At least two separate stimuli are necessary for T cell activation and the production of lymphokines, one of which acts through activation of the T cell receptor (TCR) resulting in increased intracellular calcium ($[Ca^{2+}]_i$)[6, 7] and the other that acts either through activation of protein kinase C (pkC) or through accessory cell signals such as IL-1.[8] Among the early events that result from triggering of T cell surface receptors is activation of phospholipase C, leading to hydrolysis of phosphatidylinositol 4,5-*bis*-phosphate and the generation of two important

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second messengers, inositol *tris*-phosphate and diacylglycerol. Diacylglycerol stimulates pkC activation,[9] and release of inositol *tris*-phosphate results in increased $[Ca^{2+}]_i$ through both mobilization of intracellular calcium[10] and increased membrane calcium uptake.[11] These signals communicate by ill-defined mechanisms with the cell nucleus and control a set of nuclear proteins that interact with the regulatory regions of genes such as IL-2, thus regulating transcription.

Through the use of pharmacologic agents we can manipulate $[Ca^{2+}]_i$ and pkC activation so as to investigate some of the earlier events in the T cell signal transduction cascade. Calcium ionophores directly increase $[Ca^{2+}]_i$ and can substitute for the role of TCR stimulation in T cell activation.[12] When $[Ca^{2+}]_i$ is increased, the addition of a phorbol ester such as phorbol myristate acetate (PMA), which is a potent activator of pkC,[9] results in T cell activation.[13] Depletion of T cell pkC activity by pretreatment with phorbol esters leads to the inability of these cells to proliferate in response to TCR ligands,[14] emphasizing the central role of pkC in T cell activation.

The aim of this study was to investigate the abnormalities of the T lymphocyte signal transduction pathways that lead to abnormal IL-2 production after thermal injury.

METHODS

Animal model. All animal studies were performed with the approval of and under the guidance of Harvard Medical School's Standing Committee on Animal Research and the National Institutes of Health. As previously described,[5] male A/J mice, 7 to 8 weeks old (Jackson Laboratories, Bar Harbor, Maine), were caged in groups of five in a controlled environment with water and mouse food ad libitum for acclimatization for 1 week. Animals were randomized into study groups and anesthetized with pentobarbital sodium, and their dorsa were shaved. Animals were placed in a plastic template, and a burn injury of 20% body surface area or sham burn was produced by immersion for 9 seconds in water at 90° C or room temperature, respectively. The former produced a localized, full-thickness burn.[5] Animals were resuscitated with 1 ml 0.9% saline solution, recaged under the same conditions, and killed in groups of 11 or more burns and 11 or more sham burns 4, 7, 10, 14, or 21 days later; spleens were harvested for in vitro studies.

Organ harvesting and cell culture. Spleens were teased apart, and single cell suspensions were produced. Cells were washed three times in RPMI-1640 with 2 mmol/L L-glutamine, 10 mmol/L HEPES buffer, 5×10^{-2} mmol/L 2-mercaptoethanol, and a 1% antibiotic/antimycotic solution that contained 10,000 units penicillin, 10 mg streptomycin, and 25 μg amphotericin B per ml. All reagents were obtained from Grand Island Biological Company, Grand Island, New York. The mononuclear cells were counted in Turk's solution and diluted to 2×10^6/ml in the described medium with added 5% heat inactivated fetal calf serum (complete medium). For measurement of IL-2 production and IL-2 mRNA expression, cells were cultured on 96-well microtiter plates with 200 μl per well of a solution containing 1×10^6 cells/ml at 37° C in 5% CO_2 and stimulated in one of two ways. Con A (Sigma Chemical Co., St Louis, Mo.) was used at a final well concentration of 2.5 μg/ml Con A or, alternatively, cells were stimulated with a combination of the phorbol ester, PMA (Sigma Chemical Co.) at 20 ng/ml and the calcium ionophore A23187 (Sigma Chemical Co.) at 40 ng/ml. For measurement of IL-1β and tumor necrosis factor-α (TNF-α) mRNA expression cells were stimulated with *Escherichia coli* 026:B6 lipopolysaccharide (1 μg/ml) (Sigma Chemical Co.).

Measurement of proliferative response. After 30 hours of stimulation 1 μCi tritiated thymidine (^{3}HTdr; New England Nuclear, Boston, Mass.) was added to each well, and plates were rapidly frozen 18 hours later. Plates were subsequently harvested with a multiautomated sample harvester (Cambridge Technology, Cambridge, Mass.), and ^{3}HTdr incorporation was counted for 1 minute in a liquid scintillation counter (LKB Instruments, Gaithersburg, Md.). Mitogen responses were calculated by subtracting ^{3}HTdr incorporation in unstimulated cell cultures from that in mitogen stimulated cultures.

IL-2 bioassay. Splenocytes from individual mice were cultured and stimulated with Con A or PMA/A23187, as described above. At 48 hours supernatants were harvested and frozen at −20° C for later assay. Supernatants were diluted from 1:2 to 1:128 in 100 μl complete medium and incubated for 1 hour at 37° C and 5% CO_2, and CTLL-2 cells were added (100 μl at 5×10^4 cells/ml). Cultures were incubated for 20 hours at 37° C in 5% CO_2, and proliferation was assessed by uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co.). MTT was added (125 μg per well), and after 4 hours cells were solubilized by the addition of 10% acidified sodium dodecyl sulfate (SDS). Uptake and conversion of MTT to formazan was determined in an automated enzyme-linked immunosorbent assay reader (Molecular Devices, Mountain View, Calif.) at 570 nm, with 650 nm as reference. IL-2 production was then calculated from standard curves with probit analysis (computer software provided by Brian Davis, Immunex Corp, Seattle, Wash.).

RNA isolation and Northern blot analysis. Northern blot analysis for cytokine mRNA expression was performed on days 4 through 14 after injury. After 8

Table. In vitro proliferation in mice in response to stimulation with Con A or PMA + A23187 expressed in counts per minute

	Day after burn				
	4	7	10	14	21
Con A					
Sham	40,864	51,029	47,154	78,281	89,409
Burn	20,499	31,904	36,189	74,238	78,978
Percent change	−50*	−37*	−23	−5	−12
PMA/A23187					
Sham	80,878	47,148	96,966	59,536	95,051
Burn	73,807	42,156	82,896	50,841	82,404
Percent change	−9	−11	−17	−15	−15

Percent change is the percent difference in burns compared with controls.
*$p < 0.05$.

hours of cell culture and appropriate stimulation, cells from mice of each study group ($n \geq 11$ per group) were pooled, lysed in guanidium isothiocyanate, and stored at −70° C. Total cellular RNA from 1×10^8 cells per sample was isolated by use of a phenol-chloroform extraction. RNA samples were electrophoresed on 1% agarose-formaldehyde gels and transferred to nylon membranes by capillary action. RNA was fixed to membranes by ultraviolet cross-linking.

cDNA probes. cDNA probes were produced from plasmids for murine IL-2,[15] murine IL-1β and TNF-α, and chicken β-actin and radiolabeled with [α-^{32}P] deoxycytidine triphosphate (New England Nuclear) with random sequence hexanucleotide priming.[16] Briefly, 25 ng of the cDNA probe was denatured at 100° C for 10 minutes and then incubated with Klenow enzyme, a mixture of deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxythmidine triphosphate, and 50 μCi [α^{32}P] deoxycytidine triphosphate (Boehringer Mannheim, Indianapolis, Ind.) for 120 minutes at 37° C. The reaction was terminated with ethylenediaminetetraacetate, labeling was measured in a liquid scintillation counter (LKB Instruments), and specific activity was calculated. Specific activity ranged from 5×10^8 to 5×10^9 cpm/μg. Probes were denatured with sodium hydroxide, and fish sperm DNA was added to decrease nonspecific binding.

Hybridization. Prehybridization was at 42° C for 1 to 2 hours in a solution containing 0.1% SDS, 50 mmol/L Tris, 1 mol/L NaCl, and 1 mmol/L ethylenediaminetetraacetate in a rotating glass bottle. Radiolabeled cDNA probes (25 ng) were hybridized with the membranes for 18 hours under the following conditions: 50% formamide, 1× Denhardt's solution, 0.1% SDS, 10 mmol/L phosphate buffer, 5 × SSC (0.15 mol/L NaCl, 15 mmol/L sodium citrate), and 20 μg/ml denatured salmon sperm DNA (Sigma Chemical Co.) at 42° C. Membranes were washed four times for 30 minutes each: two washes at room temperature, 2 × SSC and 0.1% SDS, and two washes at 55° C, 0.1 × SSC and 0.1% SDS. Autoradiography was performed with Kodak XAR-5 film (Sigma Chemical Co.) and intensifying screens at −70° C. Probes were stripped from membranes in a 1% glycerol solution at 80° C for 10 minutes and hybridization with β-actin cDNA was subsequently performed.

Quantification and standardization of mRNA expression. Autoradiographs were quantified with a laser densitometer (300 Series computing densitometer; Molecular Dynamics). Signal intensity for cytokine mRNA expression was normalized with the β-actin signal and expressed as a ratio of cytokine to β-actin mRNA expression.

Statistical analysis. Control and burned animals were compared and results expressed as percent change in the burn group compared with the sham group. Proliferation and IL-2 production between the different groups were compared with the Mann-Whitney *U* test. Results were considered significant if $p \leq 0.05$.

RESULTS

IL-2 production and mitogenic response to Con A stimulation. Initially, cells from burned and sham burn animals were stimulated with the mitogenic lectin Con A, which directly activates the CD3/Ti receptor complex, mimicking the effects of antigen presentation. T cell suppression was confirmed by reduction of the proliferative response to mitogenic stimulation in burned animals by 50%, 37%, and 23% on days 4, 7, and 10, respectively, after injury (Table). Significant suppression of IL-2 production in burned mice compared with controls occurred at days 7 (40% suppression), 10 (36%), and 14 (30%) after burn injury ($p < 0.05$) (Fig. 1).

IL-2 production and mitogenic response to PMA/A23187 stimulation. To determine whether abnormalities in the early initiating events of the T cell activation pathway played a significant role in abnormal activation, cells were stimulated with PMA/A23187. By di-

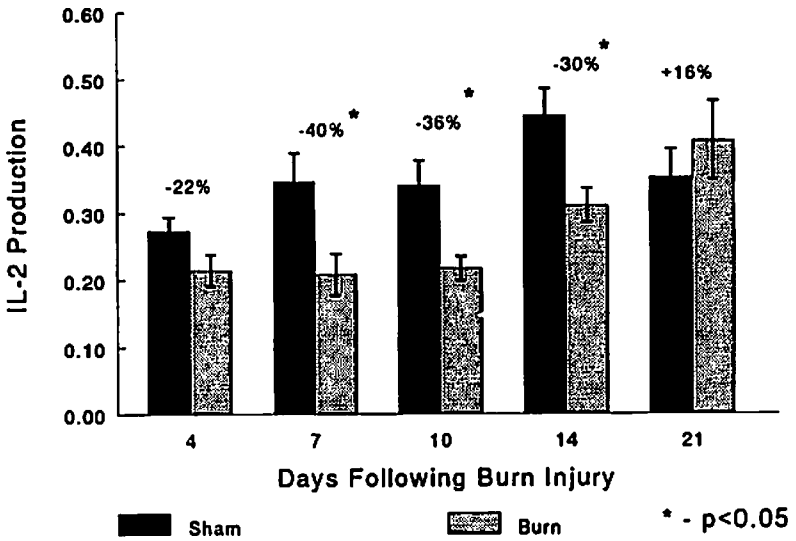

Fig. 1. IL-2 production in Con A–stimulated splenocytes of sham burn and burned animals on days 4, 7, 10, 14, and 21 after injury. Numbers above *error bars* represent percent change in burned animals compared with sham burns. $*p < 0.05$.

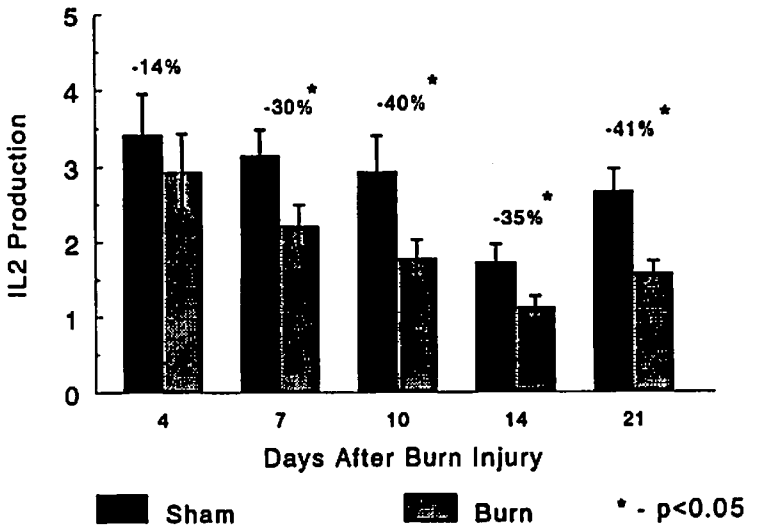

Fig. 2. IL-2 production in splenocytes of sham burn and burned animals on days 4 to 21, stimulated with PMA and the calcium ionophore A23187. Numbers above *error bars* represent percent change in burned animals compared with sham burns. $*p < 0.05$.

rectly activating pkC and increasing $[Ca^{2+}]_i$, PMA/A23187 bypasses these early events. In comparison with the sham burns, cells from burned mice showed a consistent suppression of IL-2 production when stimulated with PMA/A23187 (Fig. 2). This suppression was of a similar magnitude to and showed good temporal correlation with that seen after Con A stimulation, ranging between 30% and 41% on days 7 through 21 ($p < 0.05$, all days). The proliferative response was also consistently suppressed in burned animals stimulated in this manner, but on no day did this reach statistical significance (Table).

IL-2 mRNA expression. Having established that the abnormalities in IL-2 production after burn injury are primarily downstream of the early transmembrane signaling events, we then examined IL-2 mRNA expression to determine whether these abnormalities were pretranscriptional or posttranscriptional. Cells from burned mice, whether stimulated with Con A or with PMA/A23187, exhibited suppression of IL-2 mRNA

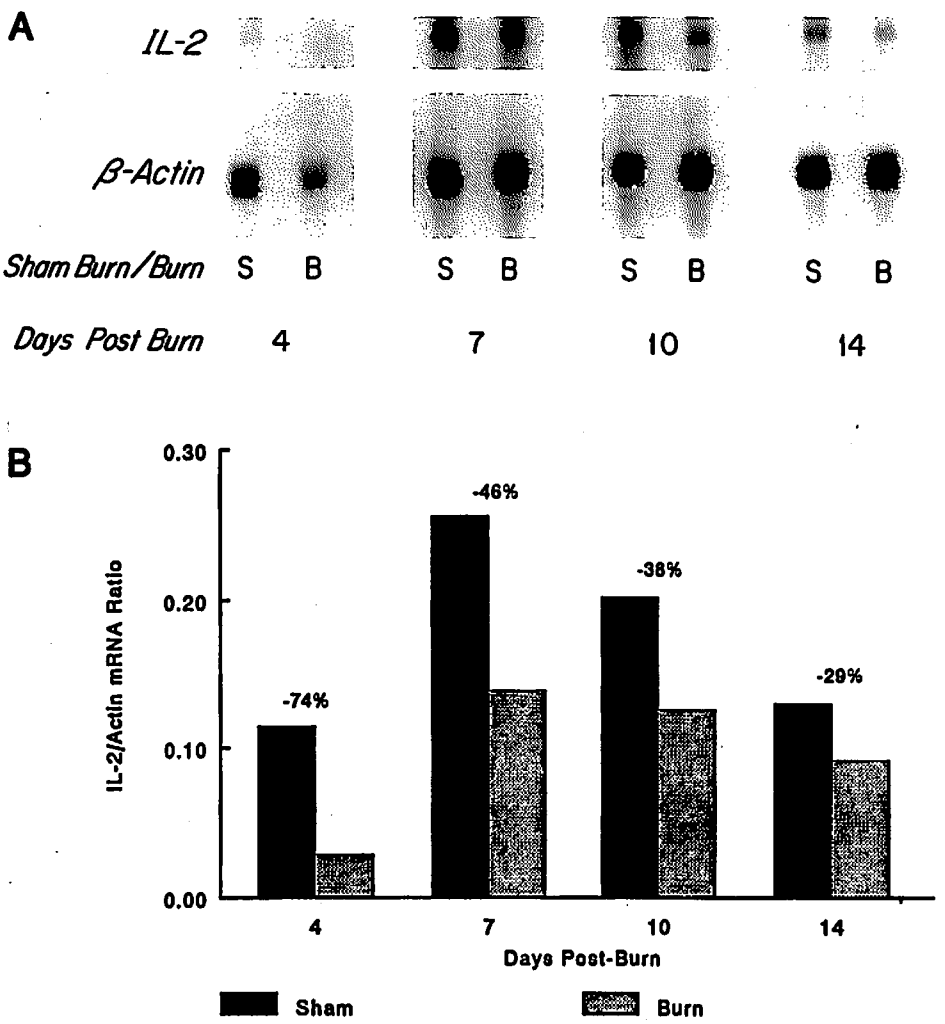

Fig. 3. A, Northern blot shows IL-2 mRNA expression in Con A–stimulated splenocytes of sham burn and burned animals on days 4, 7, 10, and 14 after injury. IL-2 message is shown *above* and β-actin control, *below*. B, Results of quantification and standardization to β-actin control of blot in *A*. Numbers above *bars* represent percent change in burned animals compared with sham burns.

expression compared with the control animals on all days (Figs. 3, *A* and 4, *A*). When quantified by densitometry and standardized to β-actin mRNA, IL-2 mRNA suppression in burned animals compared with controls was 74%, 46%, 38%, and 29% on days 4, 7, 10, and 14, respectively, for cells stimulated with Con A (Fig. 3, *B*) and 11%, 67%, 46%, and 31% on days 4, 7, 10, and 14, respectively, with PMA/A23187 stimulation (Fig. 4, *B*).

IL-1β and TNF-α mRNA expression. Expression of IL-1β and TNF-α mRNA after thermal injury were also examined to determine whether suppression of IL-2 mRNA simply represented a global suppression of cytokine expression after thermal injury. IL-1β mRNA expression was increased by 177%, 451%, 117%, and 213% in burned animals at days 4, 7, 10, and 14, respectively. TNF-α mRNA expression was suppressed by 33% on day 4 but increased on days 7, 10, and 14 by 18%, 66%, and 83%, respectively.

DISCUSSION

This study showed impaired in vitro T cell IL-2 production after burn injury, whether the CD3/Ti complex was directly activated with a mitogenic lectin or whether the early transmembrane signal transduction events were bypassed, activating cells downstream of these events with the combined use of a phorbol ester and calcium ionophore. These findings suggest that a major defect in T cell activation occurs beyond the initial membrane activating events. Although these results do not rule out additional problems in the earlier part of the pathway, the fact that the magnitude of IL-2 suppres-

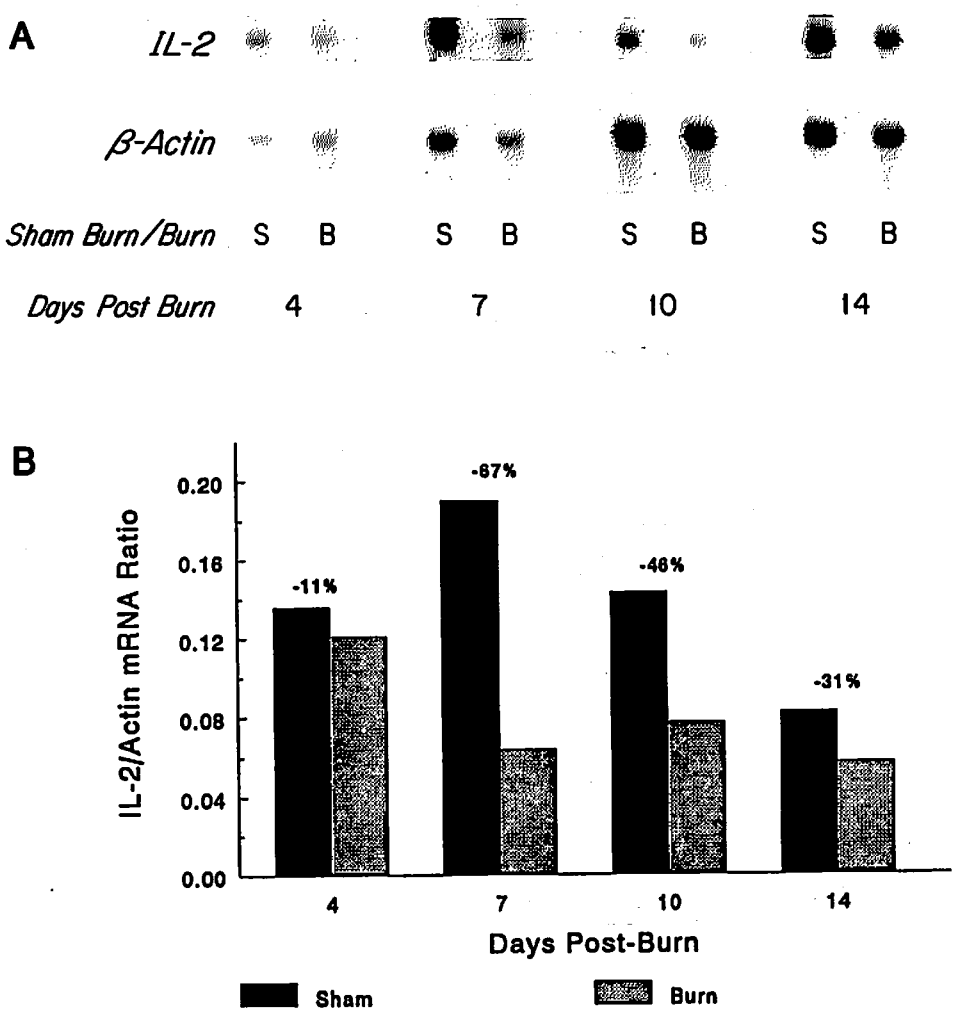

Fig. 4. **A,** Northern blot shows IL-2 mRNA expression in PMA + A23187–stimulated splenocytes of sham burn and burned animals on days 4, 7, 10, and 14 after injury. IL-2 message is shown *above* and β-actin control, *below*. **B,** Results of quantification and standardization to β-actin control of blot in *A*. Numbers above *bars* represent percent change in burned animals compared with sham burns.

sion in PMA/A23187–stimulated cells is very similar to that seen with Con A points toward the principal cellular abnormalities being at or beyond pkC activation and Ca^{2+} mobilization. These conclusions, however, make certain assumptions regarding PMA/A23187 activity. Although there is little doubt that the activation signal delivered by a calcium ionophore is an increase in $[Ca^{2+}]_i$, there is somewhat less certainty as to the identity of the signal mediated by phorbol esters. Although pkC is the only convincingly demonstrated phorbol ester receptor and the effects of phorbol esters are generally attributed to pkC activation, it is possible that the phorbol esters may also activate other signaling pathways. The situation is complicated by the fact that what we term pkC is in fact a family of pkC subtypes,[17] which we can assume to have slightly different activities. In spite of these reservations an important bank of data on pkC has been generated by the use of phorbol esters, and their usage in the investigation of these signaling pathways seems reasonable, provided that their limitations are appreciated.

In the second part of the study we attempted to further localize the molecular defects in T cells after thermal injury and, in particular, to define their relationship to IL-2 gene transcription. Whether stimulated by Con A or by PMA/A23187, a consistent and significant reduction in IL-2 mRNA expression was seen in burned animals, suggesting that blockage of IL-2 production is pretranscriptional. The fact that increases were seen in IL-1β and TNF-α mRNA expression suggests that suppression of IL-2 mRNA expression was a specific finding for IL-2 and not a result of a global suppression of cytokine mRNA expression after burn. Although we cannot rule out concomitant posttranscriptional defects,

the magnitude of IL-2 mRNA suppression is such that it is unnecessary to hypothesize further distal problems to explain the observed decrease in IL-2 production. Alternative possible explanations of our IL-2 mRNA data include alterations of cellular metabolism of IL-2 or altered kinetics of IL-2 mRNA transcription after thermal injury. Although increased cellular breakdown of IL-2 mRNA is possible, this seems less likely than a defect in regulation of gene transcription.[18] The second alternative explanation for decreased IL-2 mRNA expression in our burned animals is that the kinetics, but not the magnitude, of IL-2 mRNA expression in response to in vitro stimulation have been altered by injury. Detailed investigation of the kinetics of IL-2 mRNA expression after burn injury will be necessary to clarify this question.

Our results suggest that future investigative attempts to further define the molecular defects underlying T cell dysfunction after thermal injury should concentrate on those events from pkC activation to IL-2 mRNA transcription. The steps that follow increased $[Ca^{2+}]_i$ and pkC activation and provide the connection between the cell membrane and the nucleus are poorly understood. More information is available on the immediate activation genes and the complex nuclear regulatory molecules that control transcription of the early protein-dependent genes such as IL-2. Activation-dependent enhancer regions are known to exist in the 5′ flanking region of the IL-2 gene.[19, 20] It has been shown that all of the promoter binding sites for IL-2 must be occupied to allow activity of this enhancing region,[19, 21] suggesting that cooperative interactions between these transcriptional activators are necessary for IL-2 gene expression. One implication of the necessity for occupation of all enhancer region binding sites is that the absence of cellular signals that activate even one enhancer region may be sufficient to block gene transcription. Among the regulatory molecules that are known to interact with these promoter regions are the nuclear factor of activated T cells (NF-AT), NF-κB, Oct-1, AP-3, and the AP-1 protein complex,[19, 22] which is composed primarily of *jun* proteins[23] and regulated by *fos* proteins. The prototypes for blockage of the T cell signal transduction cascade in this region are undoubtedly the immunosuppressants cyclosporin A and FK-506, which have been shown to block transcriptional activity mediated by NF-AT, Oct-1, and NF-κB[24, 25] and have become useful pharmacologic probes for investigation of this part of the signal transduction pathway. Investigation of these nuclear factors and the immediate activation genes that encode them may further enhance our understanding of T cell dysfunction in thermal injury.

Although the precise defects that lead to abnormal T cell activation after burn injury remain unclear, this study is an early step in the identification of these abnormalities. Our results confirmed abnormal IL-2 production by stimulated T cells as a consequence of burn injury and showed that the principal cellular defects underlying this finding lie downstream of the initiating membrane events in the signal transduction pathway and before IL-2 gene transcription. Investigation of the immediate activation genes and nuclear protein molecules that regulate transcription may prove to be a fruitful area for further investigation. Identification of specific cellular and molecular events that result in T cell suppression and altered IL-2 production may ultimately lead to new diagnostic and therapeutic tools and an improved ability to recognize and counteract some of the abnormalities of cell mediated immunity that contribute to the morbidity and deaths that follow thermal injury.

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