Eur J Surg 1995; 161: 795–803

Multiple System Organ Failure May Be Influenced by Macrophage Hypoactivation as well as Hyperactivation—Importance of the Double Challenge

R. G. HOLZHEIMER, R. MOLLOY, M. V. MENDEZ, D. O'RIORDAIN, P. CURLEY, M. NESTOR, K. COLLINS, I. SAPROSCHETZ, J. A. MANNICK and M. L. RODRICK

From the Department of Surgery, Brigham and Womens Hospital, Harvard University Medical School, Boston, MA, USA

ABSTRACT

Objective: To find out if an infective challenge caused by a burn followed by caecal ligation and puncture in mice caused more abnormalities of the immune response than burn alone or caecal ligation and puncture alone.

Design: Laboratory study.

Setting: University hospital, USA.

Material: 80 male 7–8 week old A/J mice.

Interventions: Burn followed 10 days later by caecal ligation and puncture ($n = 18$), caecal ligation and puncture alone ($n = 24$), burn alone ($n = 20$), and controls ($n = 18$). The mice had their spleens removed on day 11 ($n = 28$; 6, 8, 8, and 6 in the respective groups), day 12 ($n = 26$; 6, 8, 6, and 6), and day 13 ($n = 26$; 6, 8, 6, and 6), and splenocytes and adherent cells were harvested for measurement of prostaglandin E_2 (PGE_2), interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 6 (IL-6), and tumour necrosis factor α (TNF-α).

Main outcome measures: Alterations in the production of the cytokines.

Results: After the double challenge (burn followed by caecal ligation and puncture) there were significant reductions in production of TNF-α and IL-6 compared with caecal ligation and puncture alone ($p < 0.05$), burn alone ($p < 0.05$), and controls ($p < 0.05$). These findings indicate that activation of macrophages was reduced after infection; production of TNF-α, IL-1, and IL-6 by splenocytes stimulated by lipopolysaccharide was reduced.

Conclusions: The differences do not seem big enough to indicate that mortality would be increased after caecal ligation and puncture alone. Only when there has been a previous injury (which resulted in hyperactivation of macrophages followed by a more pronounced hypoactivation) would mortality increase. In view of clinical trials with antiendotoxin and antiTNF antibodies that failed to improve survival in infected patients, we suggest that the mechanisms of the cellular immune response need further clarification.

RÉSUMÉ

But: Savoir si, chez la souris, une infection provoquée par une brûlure suivie d'une ligature et d'une ponction du caecum provoque plus de perturbation de la réponse immunitaire qu'une brûlure seule ou qu'une ligature et d'une ponction du caecum seules.

Type d'étude: De laboratoire.

Provenance: Hôpital Universitaire, USA.

Matériel: Quatre vingt souris mâles A/J âgées de 8 jours.

Méthodes: Brûlure suivi 10 jours plus tard d'une ligature et d'une ponction du caecum ($n = 18$), ligature et ponction du caecum seulement ($n = 24$), brûlure seule ($n = 20$), et contrôles ($n = 18$). Les souris ont été splénectomisées à J11 ($n = 28$; 6, 8, 8, et 6 respectivement dans chaque groupe), à J12 ($n = 26$; 6, 8, 6, et 6), et à J13 ($n = 26$; 6, 8, 6, et 6), et les cellules spléniques et les cellules adhérentes ont été recueillies pour doser les prostaglandines E_2 (PGE_2), l'interleukine 1 (IL-1), l'interleukine 6 (IL-6), et le facteur de nécrose tumorale α (FNT-α).

Principaux critères de jugement: Les variations de production des cytokines.

Résultats: Après la double agression (brûlure suivie d'une ligature et d'une ponction du caecum) les productions de FNT-α et d'IL-6 étaient réduites de manière significative par rapport à ce que l'on observait après ligature et ponction du caecum seulement ($p < 0,05$), brûlure seule ($p < 0,05$), et chez les animaux contrôles. Ces données montrent que l'activation des macrophages est diminuée après une infection; que la production de FNT-α, d'IL-1, et d'IL-6 par les cellules de la rate stimulées par les lipopolysaccharides est réduite.

Conclusions: Les différences ne semblent pas assez nettes pour montrer que la mortalité serait augmentée après ligature et ponction du caecum seule. C'est seulement lorsqu'il y a eu un traumatisme préalable (ce qui provoque une hyperactivation des macrophages suivie d'une d'ésactivation plus marquée) que la mortalité augmente. Etant donné que les antiendotoxines et les anticorps anti-FNT, utilisés dans certains essais cliniques n'ont pas permis d'augmenter la survie de patients septiques, nous pensons que les mécanismes de la réponse immunitaire de type cellulaire doivent faire l'objet d'autres études.

 ISSN 1102-4151

INTRODUCTION

Burns and other injuries may be followed by infective complications which are often lethal. Despite modern techniques resuscitation and intensive care, 80% of all deaths that take place seven days or more after multiple injuries are caused by infection (6). The pathogenesis of these infections is complex, as most organs, and both the cellular and humoral immune systems are involved (30).

Analysis of the mechanisms of the inflammatory response can best be studied in animal models. Multiple organ dysfunction is seldom the result of a single injury, for example a major burn, but is usually a "double challenge" phenomenon. A model of this phenomenon was described by Moss et al. (33) and used in our laboratory for many years. Mice are subjected to a burn which is not enough to kill them. When this is followed by an infective challenge during the 7–10 days period when the animals are immunosuppressed it results in more than 80% mortality.

Macrophages have a central role in the body's response to injury. Several investigators have discovered increased concentrations of tumour necrosis factor α, interleukin 1, and interleukin 6 after severe injuries (22, 29, 40). Macrophages are probably also responsible for increased prostaglandin E_2 production, which can suppress lymphocyte activation (16, 34, 40). It has been proposed that one of the primary inducers of this activation of macrophages may be bacterial endotoxin (41), but it is not clear whether injuries directly cause alterations in cell mediated immunity that in turn promote endotoxin and bacterial translocation or, alternatively, whether these conditions allow increased bacterial invasion that in turn inhibits cell mediated immunity. In uninfected patients alterations in the gastrointestinal mucosal barrier may allow intestinal endotoxin to enter the circulation. This response has been associated with burns (28, 43) and endotoxaemia in healthy volunteers (35).

After severe injuries there is considerable suppression of the immune response (4, 5, 31, 42). We have shown in burned mice that production of interleukin-2 (IL-2) is suppressed and the macrophage proinflammatory cytokines IL-1, IL-6 and TNFα are hypersecreted in response to stimulation with lipopolysaccharide (LPS) four to 10 days after a burn, but return to normal if the animals do not receive any further challenge (33, 36, 42). Information on cytokine production when the burn is followed by caecal ligation and puncture is, however, limited. It is not clear whether hyperactivation or hypoactivation of cytokines occurs after the second challenge, and whether this could influence treatment and the outcome of sepsis.

The aim of this study was to find out whether infective challenge caused by a burn followed by caecal ligation and puncture combined with TI, causes more abnormalities of the immune response than burn alone or caecal ligation and puncture alone.

MATERIAL AND METHODS

Animals

Eighty male 7–8 week old A/J mice (NIH Laboratories, Bethesda, Maryland) were acclimatised for one week and caged in groups of 10. They were given standard mouse food and free access to water. All animal studies were done with the approval and under the guidance of the Harvard Medical School Standing Committee on Animal Research and the National Institutes of Health.

Burn injury

Animals were anaesthetised with 60 mg/kg of pentobarbital and the dorsum was shaved. They were then subjected to a scald in a template partially submerged in water at 90°C for nine seconds, which results in a full thickness burn of 25% of the body surface area. One ml of 0.9% saline was given for resuscitation after the burn. "Sham" burn was as above except the mice were submerged in warm water.

Caecal ligation and puncture

Each mouse was anaesthetised with intraperitoneal pentobarbitone, 60 mg/kg in 1.2 ml of normal saline and the abdomen was shaved. The caecum was mobilised through a 1 cm abdominal incision and ligated at the base with a 3/0 silk tie that did not obstruct it. The caecum was then perforated through-and-through with a 27 gauge needle and a small amount of faeces extruded with gentle pressure.

Study protocol

Mice were randomised by weight into four groups: (A) burn followed by caecal ligation and puncture on day 10 ($n = 18$), (B) caecal ligation and puncture alone ($n = 24$), (C) burn alone ($n = 20$), (D) control ($n = 18$). The mice had their spleens removed on day 11 ($n = 28$; 6, 8, 8, and 6 in the respective groups), day 12 ($n = 26$; 6, 8, 6, and 6), or day 13 ($n = 26$; 6, 8, 6, and 6) and splenocytes and adherent cells were

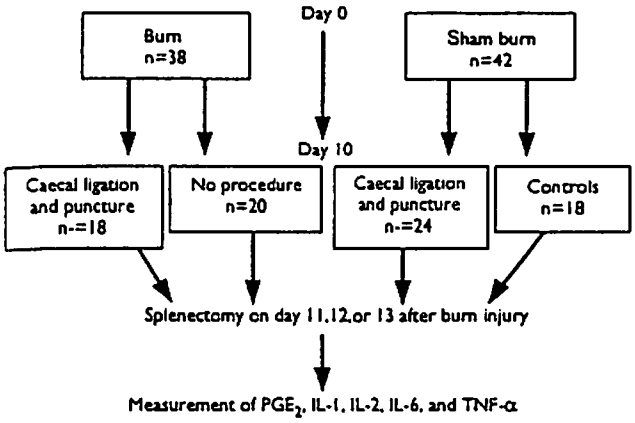

Fig. 1. Diagram of the study protocol.

harvested for measurement of concentrations of cytokines (Fig. 1).

Culture media

The complete medium was Roswell Park Memorial Institute (RPMI-)1640 containing 1% penicillin/streptomycin/amphotericin B, (10000 units/10000 μg/25 μg/ml), 1 mmol HEPES, 5×10^{-5} M 2-mercaptoethanol and 2 mmol glutamine with the addition of 5% heat-inactivated (56°C, for 30 minutes) fetal calf serum (FCS). Serum-free medium contained all additives except FCS.

Splenocytes were isolated and cultured as previously described (3, 4). Adherent cells were selected by culturing splenocytes at a concentration of 1×10^7/ml in 96-well microtitre flat-bottomed plates for one hour at 37°C in complete medium. Plates were washed three times to remove non-adherent cells, and adherent cells were cultured in serum-free medium.

IL-2 production by splenocytes

Splenocytes from individual mice were prepared at a concentration of 1×10^6/ml in complete medium and cultured in 96-well, flat-bottomed, microtitre plates in the presence of concanavalin A at end well concentrations of 2.5 μg/ml at 37°C in 5% carbon dioxide. After 48 hours supernatants were harvested and frozen at −20°C for later assay of IL-2. For bioassay of IL-2, the supernatants were diluted from ½ to 1/128 in 100 μl of complete medium, and incubated for one hour at 37°C and 5% carbon dioxide to ensure the correct pH. Cytolytic T-lymphocyte line-2 (CTLL-2) cells, after appropriate washing three times, were added to wells in 100 μl of medium at a concentration of 5×10^4/ml. Cultures were incubated for 20 hours, followed by addition of 125 μg/well of monotetrazolium. After four hours, cells were made soluble by the addition of 10% sodium dodecyl sulphate, and uptake and conversion of monotetrazolium to formazan was assayed in an automated enzyme linked immunosorbent assay (ELISA) reader (Molecular Devices, Mountain View, CA) at 570 nm, using 650 nm as reference. IL-2 production was then calculated using an IL-2 containing T cell supernatant (which was given a value of 1 unit/ml) by probit analysis.

Production of monokines by adherent cells

Adherent cells were cultured in the presence of *Escherichia coli* 026:B6 lipopolysaccharide (Sigma, St. Louis MO) at an end well concentration of 1 μg/ml. At 24 hours supernatants were removed and frozen (as above) until they were tested for monokine production.

Interleukin-6 (il-6) bioassay

Supernatants were tested for IL-6 using the IL-6 dependent cell line B9 (Genetics Institute, Cambridge, MA). Supernatants were added to wells in serial dilutions from ½ to 1/128 and plates were equilibrated at 37°C and 5% CO_2 for one hour and then 5×10^3 appropriately washed B9 cells/well were added. Plates were incubated under the same conditions for 68 hours, followed by the addition of monotetrazolium, incubated and read as above. IL-6 production in units/ml was then calculated from standard curves of recombinant IL-6 using probit analysis as for IL-2.

Tumour necrosis factor-α (TNF-α) ELISA

TNF-α was measured by a "sandwich" ELISA technique as previously described (36). Adherent cell supernatants were incubated in 96 well ELISA plates coated with hamster monoclonal anti-murine TNF-α antibody (Genzyme, Boston, MA). Plates were washed and incubated sequentially with rabbit polyclonal anti-TNF-α antibody (Genzyme), and alkaline phosphatase-conjugated goat antirabbit IgG (Boehringer Mannheim, Indianapolis, Ind., USA). The alkaline phosphatase linked antibody was then detected in an automated ELISA reader (as described above) at 400 nm after incubation with the phosphatase substrate p-nitrophenyl phosphate disodium (Sigma Chemical Co., St. Louis, Mo).

Prostaglandin E_2 (PGE_2) assay

For the quantitative measurement of PGE_2 in supernatants of adherent splenocytes stimulated with lipopolysaccharide a commercially available Radio-

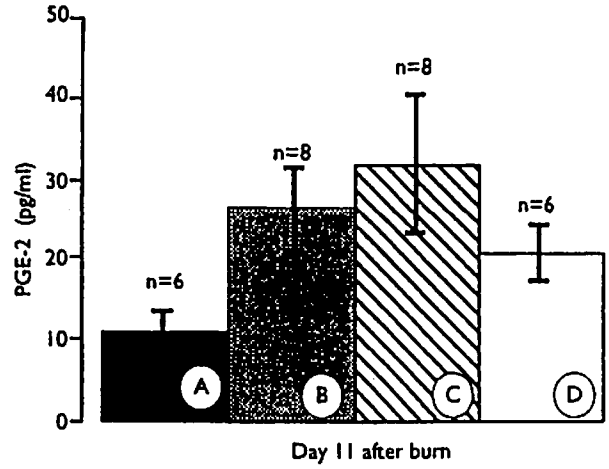

Fig. 2. Production of PGE_2 on day 11 after burn. Group A had undergone burn and caecal ligation and puncture, group B caecal ligation and puncture alone. group C burn alone, and group D acted as controls.

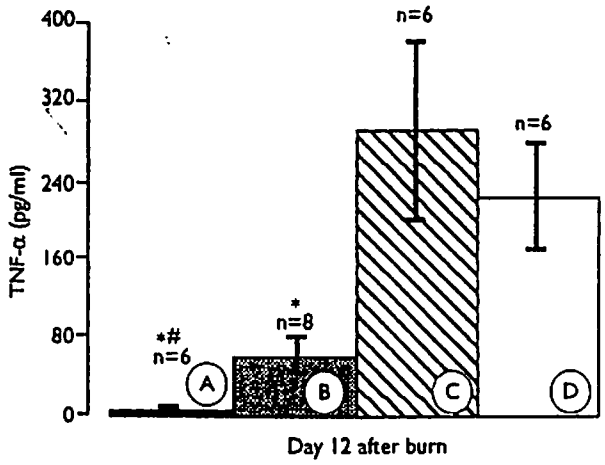

Fig. 3. Production of TNF-α on day 12 after burn. Group A had undergone burn and caecal ligation and puncture, group B caecal ligation and puncture alone, group C burn alone, and group D acted as controls. * = significantly different from group B ($p < 0.05$); # = significantly different from burn alone ($p < 0.05$) and from controls ($p < 0.05$).

immunoassay Kit (Advanced Magnetics Inc., Cambridge; MA) was used.

Statistical analysis

The differences in cytokine and PGE_2 concentrations were compared using the non-parametric Mann-Whitney test for skewed data (Instat2, GraphPad Software, San Diego CA, USA). A probability of less than 0.05 was accepted as significant. All data are presented as mean (SEM).

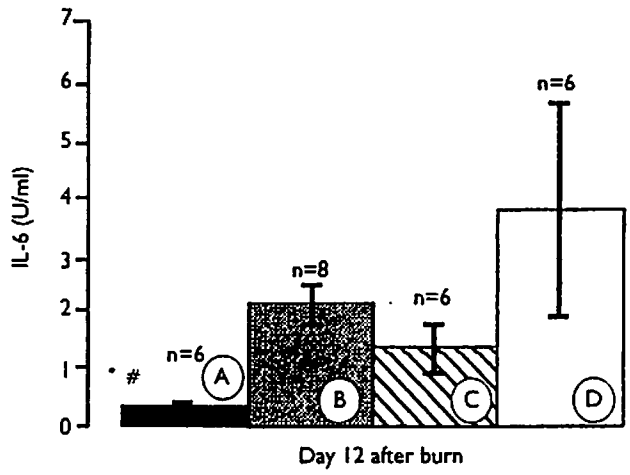

Fig. 4. Production of IL-6 on day 12 after burn. Group A had undergone burn and caecal ligation and puncture, group B caecal ligation and puncture alone, group C burn alone, and group D acted as controls. * = significantly different from group B ($p < 0.05$); # = significantly different from burn alone ($p < 0.05$) and from controls ($p < 0.05$).

RESULTS

PGE_2-production in the control group did not change during days 11, 12, or 13. PGE_2-production was slightly but not significantly suppressed on day 11 after burn in the combined group (11.7 ± 2.3 pg/ml). Neither intra-abdominal infection alone nor thermal injury alone differed from the controls (C) on any of the three days (Fig. 2).

At day 11 there was no difference in TNF-α production by the macrophages from any group. There was a significant reduction in TNF-α synthesis on day 12 in the combined group (3.5 ± 3.5 pg/ml) compared with caecal ligation and puncture alone (59.7 ± 20.4 pg/ml) or burn alone (291 ± 89 pg/ml) ($p < 0.05$) or control (225.4 ± 53.1 pg/ml) ($p < 0.05$). On day 13 after thermal injury TNFα production was significantly reduced in the combined group and the infection alone group compared with the burn alone group and the controls ($p < 0.05$). On this day, however, there was no difference in TNF production by cells from either the combined or the infection alone groups (Fig. 3).

IL-6 production was increased after burn alone (19.4 ± 7.5 U/ml) compared with controls (4.1 ± 2.1 U/ml), the combined group (3.2 ± 1 U/ml) and the infection alone group (1.9 ± 8.3 U/ml) on day 11 ($p < 0.05$). The two infection groups did not differ from the controls. On day 12 after thermal injury IL-6 production in the combined group (0.4 ± 0.03 U/ml) was significantly reduced compared with the infection alone group (2.21 ± 0.39 U/ml) ($p < 0.05$), and also the burn alone and control groups (4 ± 1.9 U/ml). On day 13 IL-6 concentrations were still significantly

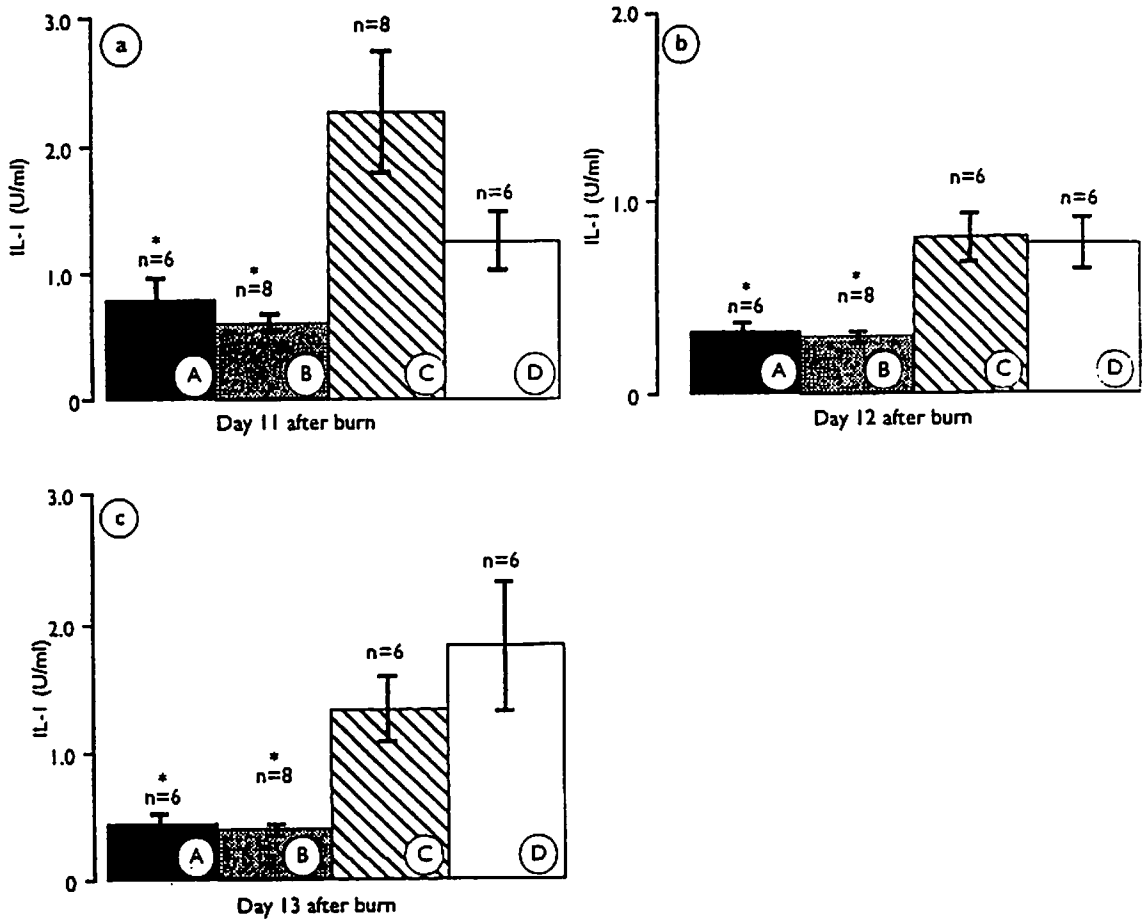

Fig. 5. (*a*) Production of IL-1 on day 11 after burn. Group A had undergone burn and caecal ligation and puncture, group B caecal ligation and puncture alone, group C burn alone, and group D acted as controls. * = significantly different from burn alone ($p < 0.01$) and from controls ($p < 0.05$). (*b*) Production of IL-1 on day 12 after burn. Group A had undergone burn and caecal ligation and puncture, group B caecal ligation and puncture alone, group C burn alone, and group D acted as controls. * = significantly different from burn alone ($p < 0.01$) and from controls ($p < 0.01$). (*c*) Production of IL-1 on day 13 after burn. Group A had undergone burn and caecal ligation and puncture, group B caecal ligation and puncture alone, group C burn alone, and group D acted as controls. * = significantly different from burn alone ($p < 0.05$) and from controls ($p < 0.05$).

less in both the infection groups than in the burn only group and the controls ($p < 0.05$) (Fig. 4).

IL-1 production was similar in the two infection groups on all three days and these concentrations were significantly suppressed compared with the burn only group and controls (Fig. 5).

IL-2 production after burn alone did not differ significantly from controls on day 11, 12, or 13, but IL-2 production by splenocytes from animals after infection alone was significantly suppressed on all three days ($p < 0.05$). IL-2 production in intra-abdominal sepsis was more suppressed on day 11 and 12 than in the combined group (Fig. 6).

DISCUSSION

These results show that there is a significant immune suppression of both adherent cell and T cell cytokine function after infection by caecal ligation and puncture. Macrophage TNF, IL-1, IL-6, and splenocyte IL-2 production were reduced by infection alone and there was an even more pronounced suppression on day 12 in the group in which a septic challenge followed the burn. Immunosuppression from the intra-abdominal infection alone does not result in increased mortality, but the combined burn and infection shows significantly increased mortality. The burn is normally associated with a mortality of 10%–20% in this model, and the mortality after burn and caecal ligation and puncture was 90%–100% on day 4 after caecal ligation and puncture (25, 33).

One of the prime functions of the immune system is to support the host's resistance to pathogens. Bacterial translocation and bacteraemia have both been associated with immunosuppression after injuries, burns, and haemorrhagic shock (3, 12, 28, 37, 39, 42).

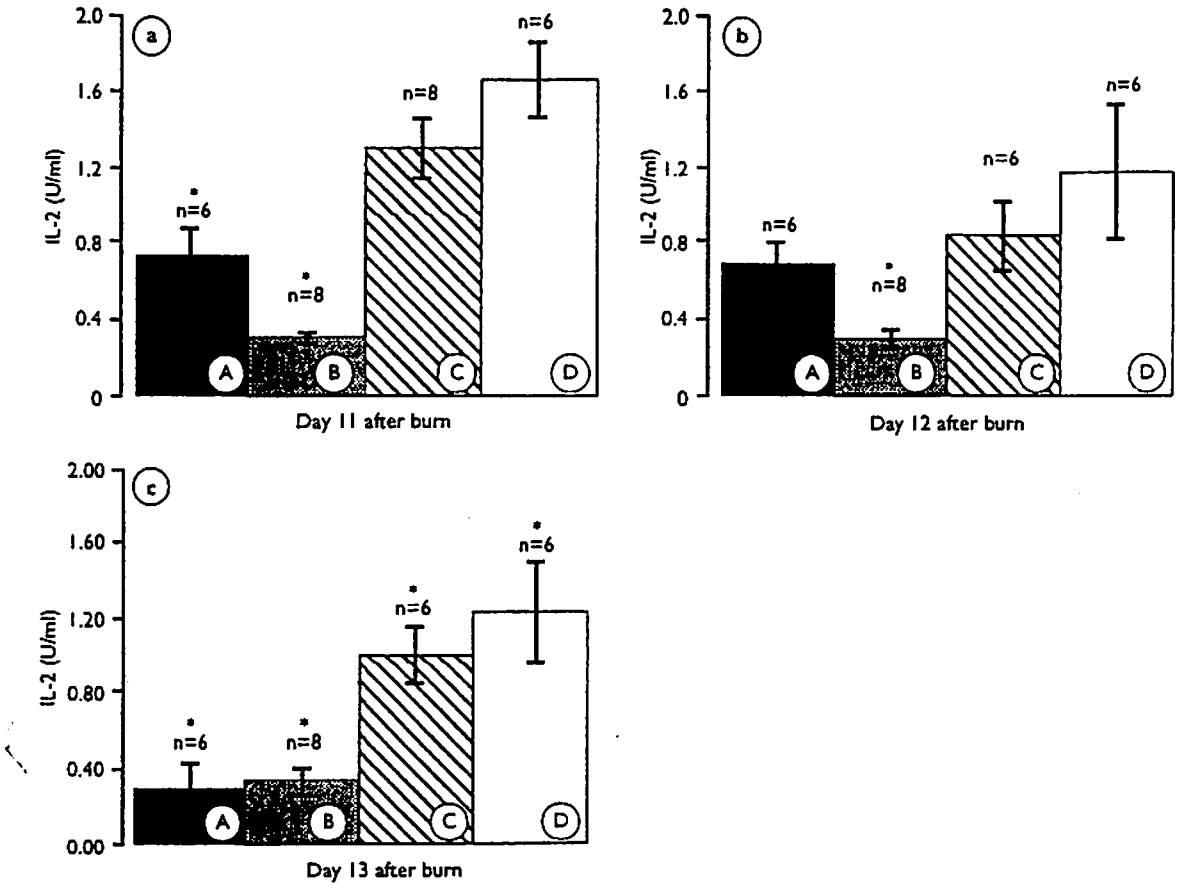

Fig. 6. (*a*) Production of IL-2 on day 11 after burn. Group A had undergone burn and caecal ligation and puncture, group B caecal ligation and puncture alone, group C burn alone, and group D acted as controls. * = significantly different from burn alone ($p < 0.05$) and from controls ($p < 0.05$). (*b*) Production of IL-2 on day 12 after burn. Group A had undergone burn and caecal ligation and puncture, group B caecal ligation and puncture alone, group C burn alone, and group D acted as controls. * = significantly different from burn alone ($p < 0.05$) and from controls ($p < 0.05$). (*c*) Production of IL-2 on day 13 after burn. Group A had undergone burn and caecal ligation and puncture, group B caecal ligation and puncture alone, group C burn alone, and group D acted as controls. * = significantly different from burn alone ($p < 0.01$) and from controls ($p < 0.01$).

Like our model of burn and intra-abdominal infection, clinical multiple organ failure is related to a double challenge phenomenon. The first event, the burn, primes the immune system so that after the second event, the infection, the immune response is altered appreciably. Hypotension, ischaemia-reperfusion injury, and hypoxaemia caused by injury are thought to prime macrophages and neutrophils. Deitch (11) showed that with repeated challenge the effects of endotoxin could be lethal in an otherwise non-lethal dose. He also suggested (13) that any further challenge to the immune system might involve hyperactivation of macrophages.

It is still not clear whether injury directly changes the cell mediated immune response, which then favours bacterial translocation, endotoxaemia and bacteraemia, or whether bacterial translocation provokes changes that lead to detrimental effects on cell mediated immunity (21). Moss et al. (33) have shown that the immune suppression of lymphocyte proliferation and IL-2 suppression after burns correlates with increased susceptibility to infection. IL-2 treatment not only improved the cell mediated immune response, but also the survival rate after burns and caecal ligation and puncture (20). Gough et al. (21) also showed that lymphocyte proliferation, which is suppressed after caecal ligation and puncture, can be restored by giving IL-2. There is evidence that injuries are accompanied by macrophage hyperactivation (36) as measured by increased TNF and IL-6 secretion compared with sham controls. Maximum TNF secretion occurred on days 7 or 10 after burn. It was not clear whether the double challenge phenomenon (burn followed by intra-abdominal infection), would

cause excessive production of cytokines or suppression. In view of current clinical studies with antiTNF antibodies or antiendotoxin antibodies this could be of fundamental importance.

Production of PGE_2, which is responsible for immune suppression, is increased after burns (32). PGE_2 production after burns and intra-abdominal infection was only moderately reduced ($p = 0.059$) on day 11 in this study. There were no significant differences on the other days. These results suggest that PGE_2 was probably not responsible for the macrophage hypoactivation seen in the two infection groups in this study.

Sequential measurements of TNF in clinical sepsis have shown that overproduction of TNF is correlated with outcome (9, 19). We found that TNF-α production was significantly reduced in the double challenge group on day 12 after the burn. According to Adams and Hamilton (2) macrophage activation is accompanied by increased production of TNF in contrast to primed macrophages, which do not respond to TNF production. The finding of reduced TNF production correlates with the reduction in the survival rate after the double challenge on days 12 and 13 as previously described (25). Despite treatment with pentoxifylline, a compound known to decrease TNF production, there was a decreased survival rate at day 12 and 13 after thermal injury. It was unclear whether this was caused by unknown factors not covered by our treatment, or due to altered macrophage function.

The tolerance to endotoxin has been associated with reduced secretion of TNF (38), but in our model the lack of response of macrophages to the secondary challenge with lipopolysaccharide did not have a protective effect. Macrophage activation is accompanied by increased phagocytosis, antigen presentation, and production of TNF and IL-1 (1, 27). Endotoxin could activate macrophages directly without activating T cells. Neither PGE_2, which is also a macrophage product, nor TNF were raised after the double challenge. It is therefore likely that macrophage hypoactivation was the consequence of the double challenge, and hypoactivation with reduced TNF production may be responsible for the increased mortality. This is supported by Echtenacher et al. (14) who showed that production of endogenous TNF is necessary for survival in a model of experimental peritonitis. Interferon gamma, which is essential for macrophage activation, is produced in optimal concentration only if IL-1 or IL-2 are present in sufficient concentration (18). We have shown that IL-1 and IL-2 production are suppressed in both the infection groups. Despite the fact that there was no significant difference between IL-1 production in the double challenge and peritonitis groups, and that IL-2 suppression was even more pronounced in the peritonitis group on days 11 and 12, survival after the double challenge was less than survival after intra-abdominal infection alone. It seems likely that in addition to the observed cytokines (IL-1 and IL-2) other factors could be involved in the immune suppression after the double challenge.

TNF causes the partial release of IL-6 after endotoxin has been given. As antiTNF antibodies do not totally suppress IL-6 production, other factors such as IL-1 could be important in the production of IL-6 (15). In this study IL-6 macrophage production was significantly reduced after the double challenge. Several clinical studies have suggested that high serum concentrations of IL-6 are associated with death from infection (8, 10, 23). It was not clear whether IL-6 was directly responsible for the death or was acting like an "alarm" hormone. In this study we have shown for the first time that macrophage IL-6 production is reduced after a double challenge. Together with IL-1, IL-6 is involved in activation of T-cells (26). While IL-1 is influencing the induction of IL-2 production, IL-6 regulates the optimal IL-2 reactivity (24). Increased IL-1 production in endotoxin-resistant mice correlated with increased survival rate after caecal ligation and puncture (7). Faist et al. (17) showed that the IL-1 production of peripheral monocytes after injury and sepsis was suppressed, and concluded that this was partly the result of increased production of PGE_2. Our observation that IL-1 production is reduced after double challenge and peritonitis confirms this finding, and we have also shown that PGE_2 production does not seem to be the cause of immunosuppression after double challenge.

Intra-abdominal infection alone is accompanied by suppression of IL-2 production (21). The authors speculated that sepsis and injury have similar consequences, as in both cases there is a reduction of IL-2 production. In our study, however, IL-2 production was reduced in both infection groups, but mortality was increased only after the double challenge.

In summary, macrophage activation is reduced after infection with a simultaneous reduction in the production of TNF-α, IL-1, and IL-6 by splenocytes stimulated by lipopolysaccharide. This does not seem to be enough to increase mortality after caecal ligation and puncture alone. Only when there has been a previous challenge such as a burn (which results in hyperactivation of macrophages followed by a more pronounced hypoactivation) would mortality increase. In view of clinical studies with antiendotoxin antibodies or antiTNF antibodies, which have so far failed to improve survival in infected patients, the mechanisms of the cellular immune response to sepsis need further clarification.

ACKNOWLEDGEMENTS

We thank Dr. Brian Davis of Immunex Corp., Seattle, WA, USA, for providing the computer software for probit analysis, and Dr. Lucien Aarden of the Netherlands Red Cross Transfusion Service for giving permission to us to use the IL-6 dependent cell line B9.

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Submitted September 15, 1994; accepted November 28, 1994

Address for correspondence:
Dr René G. Holzheimer
Chirurgische Universitätsklinik
Josef-Schneider-Str. 2
D-97080 Würzburg
Germany