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Effect of low dose recombinant interleukin 2 plus indomethacin on mortality after sepsis in a murine burn model

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Under anaesthesia, 129 8-week-old male A/J mice were subjected to a 25 per cent scald or sham burn and then resuscitated. They were divided at random into two groups. Mice from the first group were allocated into two groups. Mice from the first group were allocated into four subgroups to receive 6 days intraperitoneal (I.P.) injections as follows: (i) recombinant human interleukin 2 (rhIL-2) (250 units day^{-1}); (ii) saline; (iii) indomethacin (5 μg^{-1} day^{-1}); or (iv) rhIL-2 (250 units) + indomethacin (5 μg). Sham burned mice served as no treatment controls. All animals were subjected to peritonitis induced by caecal ligation and puncture 10 days after the burn and mortality was assessed. Mice from the second group were allocated to two subgroups to receive 6 days intraperitoneal injections of: (i) rhIL-2 + indomethacin; or (ii) saline. Animals in this group did not undergo septic challenge. They were randomly killed on days 7, 9 or 10 after the burn. Their splenocytes were harvested and assayed for response to the mitogens phytohaemagglutinin (PHA) and concanavalin A (Con A), and for production of interleukin 2. Mortality rate in animals subjected to burn and septic challenge without treatment was 75 per cent; in mice receiving rhIL-2 alone it was 68 per cent, in mice receiving indomethacin alone it was 62 per cent (no significance) and in mice receiving rhIL-2 + indomethacin it was reduced to 38 per cent ($P < 0{\cdot}02$). Splenocytes from animals receiving combination therapy had markedly improved responses to PHA on days 7 ($P = 0{\cdot}01$), 9 ($P = 0{\cdot}02$), and 10 ($P = 0{\cdot}008$), and to Con A on days 7 ($P = 0{\cdot}001$), 9 ($P = 0{\cdot}002$) and 10 ($P = 0{\cdot}001$), after burn injury. Interleukin 2 production was also significantly ($P = 0{\cdot}004$) improved by therapy with rhIL-2 + indomethacin. These data suggest that low dose rhIL-2 in combination with indomethacin may have potential use in the therapy of burn victims.

Keywords: Interleukin 2, burns, therapy

Death from sepsis with resultant multiorgan failure continues to be a major problem in dealing with patients following trauma. Baker *et al.* in 1980 reported that, of deaths occurring later than 7 days after injury, 78 per cent were due to sepsis[1]. In thermally injured patients 50–70 per cent of deaths are due to sepsis[2,3]. Donati *et al.* noted in 1983 that, if burn patients developed significant infection, the mortality rate rose to 80 per cent[4]. In separate reports Alexander *et al.*[5] and Casson *et al.*[6] studied the host defences in burn patients and found them to be compromised. Markley, in 1968, reported finding positive blood cultures in over half of thermally injured mice, most due to Gram-negative organisms[7]. Many authors have elucidated the specific immune defects after a burn, including defective neutrophil function[8], decreased lymphocyte blastogenesis in response to mitogens[9], changes in T cell subpopulations[10,11], and the presence of circulating immunosuppressive factors[12–15].

Impairment of interleukin 2 (IL-2) production has been demonstrated after a burn and is prolonged after T cell subpopulations have returned to normal[16]. It was therefore a logical step to attempt to restore immunocompetence after thermal injury by treatment with exogenous IL-2. We have previously reported the use of recombinant human interleukin 2 (rhIL-2) therapy in a murine burn sepsis model[17]. In these experiments, following a 25 per cent scald injury, rhIL-2 was injected by the intraperitoneal route for 6 days, and after 3 days of rest the animals were subjected to a septic challenge of peritonitis induced by a caecal ligation and puncture (CLP) procedure. The mortality rate was significantly reduced by IL-2 therapy. However, the dose used in this work, when adjusted for human therapy, is known to be associated with undesirable toxic side effects[18] and limits its usefulness for human application. The aim of this study was to assess the efficacy of a low dosage of rhIL-2 in treating burned animals which could, if effective, be used in human therapy.

Furthermore, it has also been shown by others that prostaglandin E_2 (PGE_2) inhibits IL-2 production by human cells[19]. We have shown that the cyclo-oxygenase pathway is probably involved in the mechanism of post-burn immunosuppression[20]. Therefore, we also assessed the use of the prostaglandin inhibitor indomethacin, alone and in combination with rhIL-2, as effective immunomodulators.

Materials and methods

Burn model

Male A/J mice, 7 weeks old, were obtained from Jackson Laboratories, Bar Harbor, Maine, USA. They were acclimatized for 1 week in the animal house under controlled conditions with water and mouse chow *ad libitum*. The mice were randomized into burn and sham burn groups, and were anaesthetized with pentobarbitone (1·25 mg mouse^{-1} in 0·25 ml saline) by intraperitoneal injection. The animals were shaved,

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over the dorsum and placed in a specially constructed mould exposing 25 per cent body surface area. Immersion of the mould in water at 90°C for 9 s resulted in a histologically proven full thickness burn. Resuscitation with 1 ml saline followed and the mice were caged in groups of five. Sham burn involved anaesthesia and shaving only. This protocol and all animal procedures were approved by the Standing Committee on Animals, Harvard Medical Area, under NIH guidelines.

Septic challenge

Under pentobarbitone anaesthesia the abdomens were shaved, opened, the caecum located and ligated at its base with 0/0 silk (Ethicon, Somerville, New Jersey, USA). The caecum was then punctured through and through with a 27 gauge needle and faeces expressed to ensure peritonitis. The abdomens were closed with 5/0 Ethilon® (Ethicon). Mortality was assessed at 96 h after CLP as this laboratory has consistently found maximum mortality occurs by this time using this model.

T cell mitogen responses

Splenocytes were suspended in RPMI 1640 medium with 2 mM l-glutamine, 5×10^{-5} M 2-mercaptoethanol, 1 per cent antibiotic antimycotic (penicillin 10 000 units, streptomycin 10 000 μg, amphotericin 2·5 $\mu g\,ml^{-1}$), gentamicin (40 $\mu g\,ml^{-1}$) and 10 mM HEPES (all reagents for cell washing and culture were obtained from Grand Island Biological Co., Grand Island, New York, USA). Cell suspensions were washed three times in this medium, centrifuging at 1500 r.p.m. for 10 min. Mononuclear cells were counted using Turk's solution and cultured in microtitre plates at 2×10^5 cells $well^{-1}$ in 200 μl medium with 5 per cent heat-inactivated fetal calf serum (56°C, 30 min), with or without phytohaemagglutinin (PHA) at 5 $\mu g\,ml^{-1}$ or concanavalin A (Con A) at 1·25 $\mu g\,ml^{-1}$. Assessment of cell viability was made using trypan blue and was always >95 per cent. The plates were incubated at 37°C in 5 per cent CO_2 for 24 h. At 24 h the plates were pulsed with 1 μCi $well^{-1}$ of tritiated thymidine (^{3}HTdr) (New England Nuclear, Boston, Massachusetts, USA), and following a further 18 h of incubation the plates were frozen. Cells were harvested in a multi-automated sample harvester (Cambridge Technology, Cambridge, Massachusetts, USA). Incorporation of ^{3}HTdr was measured in a liquid scintillation counter (LKB Instruments, Gaithersburg, Maryland, USA).

Production and assay of interleukin 2

Splenocytes from individual mice were harvested, washed, counted as above, and adjusted to 2×10^6 cells ml^{-1}; 100 μl $well^{-1}$ of these cells were placed on 96 well microtitre plates and incubated with Con A 1·25 $\mu g\,ml^{-1}$ for 48 h. Supernatants were carefully withdrawn using a Pasteur pipette and frozen. For IL-2 assay the supernatants were thawed and diluted from 1:2 to 1:128 in the above medium with a 100 μl volume, and incubated for 1 h at 37°C in 5 per cent CO_2. CTLL-2 cells were washed free of T cell growth factor three times, diluted to 5×10^4 ml^{-1} and 100 μl added to each well. Cultures were incubated for 20 h, pulsed with ^{3}HTdr, harvested 4 h later and counted to assess uptake of ^{3}HTdr. A standard mouse IL-2 pool was given a value of 1 unit. IL-2 production was determined by comparison with this standard unit by probit analysis[21] with the aid of a computer program kindly provided by Dr Brian Davis. Suppression of IL-2 was calculated using the formula:

$$\% \text{ Suppression} = 100\left(1 - \frac{\text{mean IL-2 production in units for each individual mouse}}{\text{mean IL-2 production in units for the control group}}\right)$$

Treatment protocol

One hundred and twenty-nine mice were subjected to either a burn (112 mice) or sham burn injury (17 mice). They were randomly allocated to one of two major experimental groups. The first group was used to investigate the effects of various immunomodulating therapies on the mortalities after scald injury and subsequent septic challenge. This group consisted of 64 burned and 8 sham burned mice. The burned mice were further allocated to one of four treatment groups of 16 mice each. Treatment groups received by daily i.p. injection (beginning 1 day after burn injury) either: (i) 250 units rhIL-2 + 5 μg indomethacin $mouse^{-1}$ (Merck and Co., West Point, Pennsylvania, USA), (ii) saline, (iii) 5 μg indomethacin, or (iv) 250 units rhIL-2. After 6 days of therapy the mice were allowed to rest for 3 days, then subjected to septic challenge by CLP as above. Sham burned mice received no therapy but were subjected to CLP.

The second experimental group was used to determine if the effects on mortality noted in the first part of the experiment were reflected by changes in the responses to the mitogens PHA and Con A, and in the production of IL-2. This group consisted of 48 burned mice allocated to one of two equal-sized treatment groups. One group received 250 units rhIL-2 + 5 μg indomethacin $mouse^{-1}$ day^{-1}. The second group received intraperitoneal injections of saline. All mice were treated for 6 days. All injections were 1 ml in volume. Eight mice from each group were killed in a CO_2 chamber on days 7, 9, and 10 post-burn, their splenocytes harvested and mitogen responses assessed. Nine sham burned mice were untreated.

Statistical analysis

Statistical analysis was made using Student's t test for PHA, Con A, and IL-2 data and Fisher's exact test for survival data, using the computer program Tadpole (Biosoft, Milltown, New Jersey, USA). Significance was assumed at the 95 per cent confidence level.

Results

Survival after burn and sepsis

Sham burned animals had no mortality after sham burn and a 20 per cent mortality rate when subjected to CLP. In animals receiving a 25 per cent body surface area burn and subjected to CLP 10 days later, the mortality rate was 75 per cent. Therapy with low dose rhIL-2 alone (250 units) reduced the mortality rate slightly to 68 per cent (not significant). Similarly, treatment with indomethacin alone further reduced the mortality rate to 62 per cent, but again not reaching significance. However the combination of rhIL-2 plus indomethacin resulted in a significant reduction of mortality rate to 38 per cent ($P<0·02$).

Restoration of in vitro *mitogen responses after* in vivo *therapy*

When the mitogen responses after burn and after therapy were studied, it was found that there was significant improvement in the responses of splenocytes from the rhIL-2 + indomethacin-treated mice to PHA on days 7 ($P=0·01$), 9 ($P=0·02$) and 10 ($P=0·008$) post-burn compared with saline-treated animals (*Figure 1a*). The greatest enhancement (100 per cent) of PHA response was found on day 10 (*Figure 1b*).

There were similar improvements in responses to Con A after therapy with rhIL-2 + indomethacin on days 7 ($P=0·001$), 9 ($P=0·002$) and 10 ($P=0·001$) (*Figure 2a*). The greatest enhancement of Con A response was found on day 7 post-burn (>100 per cent) (*Figure 2b*). In sham burned mice, overall responses to mitogens were surprisingly not significantly different from saline-treated burned animals, although in many individual measurements burned mice were indeed immunosuppressed.

The production of IL-2 post-burn and therapy was also examined, and it was found that on day 7 post-burn, saline-treated mice showed significantly suppressed IL-2 production ($P=0·0001$) compared with sham burned animals (*Figure 3*). Furthermore, therapy of burned mice with low dose rhIL-2 + indomethacin resulted in a significant increase in IL-2 production ($P=0·004$) compared with saline-treated burned mice.

Discussion

The results reported here demonstrate that the combination therapy of low dose rhIL-2 with indomethacin is able to improve cell-mediated immunity in burned mice, measured *in vitro*, and is associated with a significantly improved survival rate in burned mice subjected to septic challenge by CLP.

Many authors have attempted to abrogate the immunosuppression observed in thermally injured animals with a variety of immunomodulators including: vitamin A[22], *Corynebacterium parvum*[23], polymyxin B[24] and cyclophosphamide[25]. Zapata-Sirvent *et al.* reported improved survival rates with specific pharmacological therapy in burned mice in dosage regimens shown to improve cell-mediated immunity, when the animals were subjected to subsequent infectious challenge[25]. These drugs included cimetidine, cyclophosphamide, ibuprofen and cerium nitrate. It should be noted, however, that there were no controls used in the experiments for either vehicle or fluid volume, which in themselves are likely to affect survival after burning.

Hansbrough *et al.*[26] showed improvement in post-burn

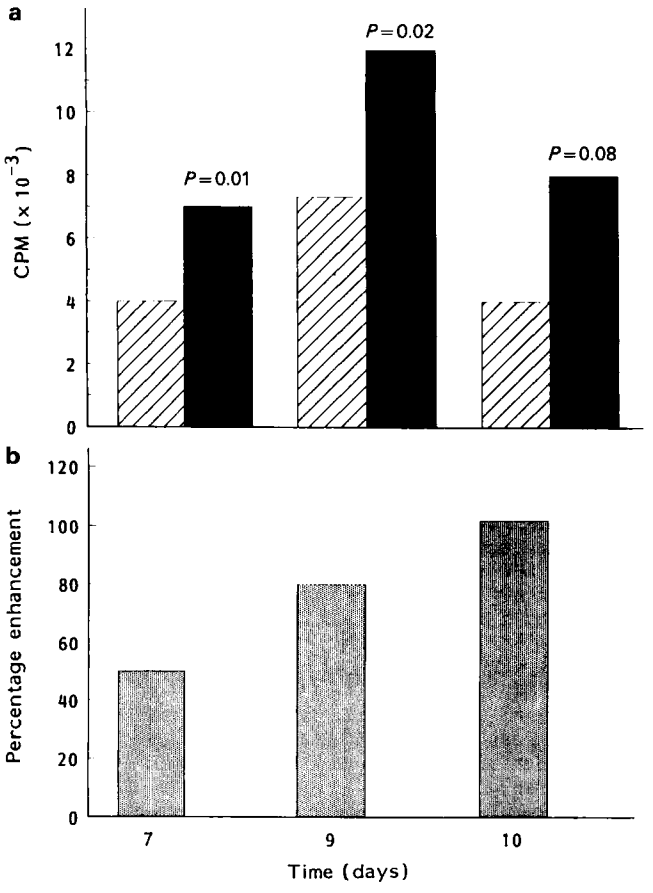

Figure 1 **a** *Responses to phytohaemagglutinin (PHA) by splenocytes from burned mice treated with saline (▨) or interleukin 2 + indomethacin (■). P values expressed as compared with saline-treated mice (n = 8 for each group on each day).* **b** *Percentage enhancement of responses to PHA post-therapy with interleukin 2 + indomethacin*

cell-mediated immunity using the prostaglandin inhibitors ibuprofen and indomethacin, but this animal model did not include an infectious challenge. Waymack *et al.*[23] in their burn model included a single injection of bacteria into the peritoneal cavity and reported improved survival rates after administration of pharmacological agents, including *C. parvum* and TP-5. However, a single dose of bacteria does not resemble the continuous infectious challenge that occurs in thermally injured patients, which is probably more nearly duplicated by the CLP model used in the present study.

Over recent years the role of prostaglandins in the regulation of the immune response has been studied. They have been shown to be immunosuppressive probably by activation of suppressor cells[27,28]. The release of large amounts of prostaglandins of the E series as a direct consequence of traumatic and burn injuries is well documented[29]. Many authors have reported on the immunomodulating effects with prostaglandin synthetase inhibitors. Indomethacin has been shown to reverse depressed cellular immunity seen with Hodgkin's disease[30], and it corrected immunosuppression and slowed tumour growth in mice with chemical and virus-induced tumours[31]. Webb and Jamieson described a mouse spleen cell which suppressed the PHA and Con A response of normal mouse spleen cells; this suppression was blocked by indomethacin and stimulated by PGE_2[32]. It has been shown that post-burn immunosuppression seen in a mouse model could be abrogated by removal of burn eschar or infusion of peritoneal macrophages from unburned mice. The authors went on to suggest that it was the presence of prostaglandin-producing macrophages that led to the observed immunosuppression[33].

Miller *et al.* suggested that the presence of prostaglandin-producing macrophages is a normal response in a local wound, and that it is the quantity of these inhibitory-suppressor macrophages that is the important factor in host defence[34]. The implication is that thermal trauma results in local immunosuppression, and with sufficient injury spills over and initiates harmful systemic effects constituting systemic immunosuppression[34]. One of the major inhibitors of IL-2 production in human cells is PGE_2. PGE_2, a product of the cyclo-oxygenase pathway, is increased in burn patients, and circulating lymphocytes in these patients have been shown to be more sensitive to its effects than those from normal individuals[35,36].

IL-2 induces T cell proliferation, induces IL-2 receptors, enhances production of T cell growth factor and γ-interferon, and induces T cells to become cytotoxic[37]. In large doses (>90 000 units kg^{-1} day^{-1}) IL-2 causes significant side effects, e.g. fever, chills, nausea, vomiting, tachycardia and general

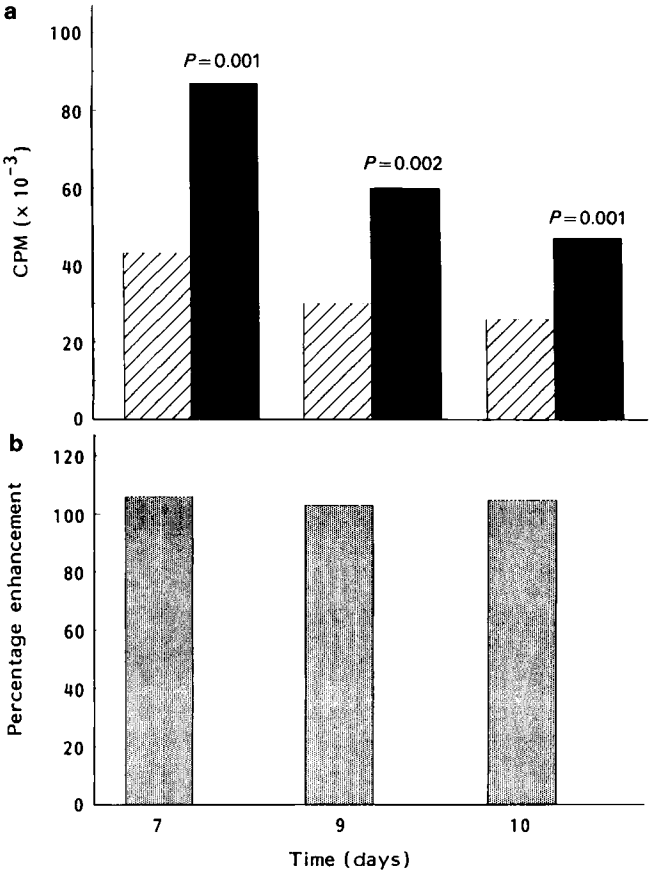

Figure 2 **a** *Responses to concanavalin A (Con A) by splenocytes from burned mice treated with saline (▨) or interleukin 2 + indomethacin (■). P values expressed as compared with saline-treated mice (n = 8 for each group on each day).* **b** *Percentage enhancement to responses to Con A post-therapy with interleukin 2 + indomethacin*

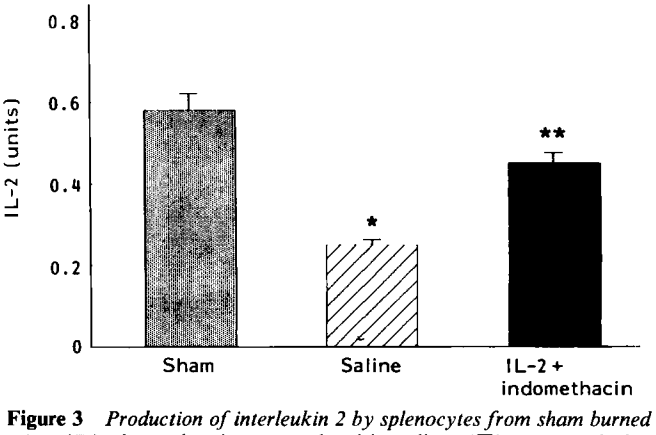

Figure 3 *Production of interleukin 2 by splenocytes from sham burned mice (▩), burned mice treated with saline (▨) or interleukin 2 + indomethacin (■). *P = 0·001 compared with sham burned mice; **P = 0·004 compared with saline-treated mice*

malaise[37]. We have previously shown in the same burn sepsis model that rhIL-2 (16000 units day^{-1}) improves survival compared with untreated animals[17]. A dose of 250 units day^{-1} also showed beneficial effects on survival but this benefit did not reach significance. Gough *et al.*[17] showed that mouse spleen cells were more responsive to mitogen stimulation with PHA and Con A when IL-2 was added *in vitro*, and went on to suggest that IL-2 had improved T cell function by increasing IL-2 receptor expression. This group also noted that endogenous IL-2 production was suppressed by *in vitro* IL-2 therapy when compared with controls. However, the data presented in this report indicate that endogenous production of IL-2 is increased after therapy with low dose rhIL-2 in combination with indomethacin, possibly accounting for the increased mitogen responses and improved survival rates seen in the present experiments. The dose of rhIL-2 used in this report (i.e. 250 units day^{-1} or 10000 units kg^{-1} day^{-1}), when adjusted for human therapy, is less than the doses used *in vivo* by investigators such as Rosenberg *et al.*[38] in cancer therapy. Furthermore, the dose of indomethacin when converted to man units by weight would be equivalent to 14 mg day^{-1}, a very low dose and likely to be without toxicity even in thermally injured patients.

We feel that this therapy may have clinical therapeutic usefulness in reducing mortality after sepsis in thermally injured patients.

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