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Long-Term Immunotherapeutic Intervention with Pentoxifylline in a Mouse Model of Thermal Injury and Infection

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Major thermal or traumatic injury often results in abnormalities of immune function, and these abnormalities contribute to the increased susceptibility to infection observed in these patients. Abnormalities of T-cell function, including decreased proliferation and secretion of cytokines are observed following major injury and, conversely, there is markedly increased monokine production. Thus, therapy of this syndrome might logically be aimed at modulating the immune system to upregulate T-cell function and downregulate monocyte hyperactivation. Pentoxifylline (PTX), a methylxanthine derivative, has been shown to be therapeutically effective in several animal models.

The purpose of this study was to evaluate PTX and its effect on cytokine production in a mouse model of thermal injury and to study its effect on survival after septic challenge.

The results show that PTX therapy after injury can restore T-cell production of IL-2 and downregulate the hyperactive macrophage secretion of proinflammatory cytokines. However, improvement in survival resulting from this therapy following thermal injury and septic challenge depends on timing of dosage.

Major thermal or traumatic injury often results in abnormalities of immune function, and these abnormalities contribute to the increased susceptibility to infection observed in these patients. Abnormalities of T-cell function, including decreased proliferation and secretion of cytokines, are observed following major injury and, conversely, there is markedly increased monokine production. Multiple system organ dysfunction (MSOD) is often found after these episodes and has been suggested to be triggered at least in part by endotoxemia of endogenous or exogenous origin.[1] Immune abnormalities have been correlated with infection and MSOD.[1–4] Thus, therapy of this syndrome might logically be aimed at modulating the immune system to upregulate T-cell function and downregulate monocyte hyperactivation.

Pentoxifylline (PTX), a methylxanthine derivative, has been shown to be therapeutically effective in several animal models. In hemorrhagic shock PTX restored microvascular blood flow,[5] increased hepatic surface oxygen tension,[6] improved tissue oxygenation and consumption,[7] and resulted in increased survival.[8] PTX also produced a positive effect in acute respiratory distress syndrome (ARDS),[9] an attenuation of acute lung injury in sepsis,[10,11] and likewise improved pulmonary hemodynamics during acute hypoxia.[12]

In animal models of acute peritonitis PTX improved hemodynamic and histological changes, decreased neutrophil adhesiveness,[13] attenuated pulmonary leucostasis, pulmonary capillary occlusion, and endothelial and epithelial damage.[14] PTX reduced mortality in a sepsis model[15] and diminished multiple organ albumin leak.[16] PTX increased survival of mice after endotoxic shock[17] and decreased TNF production.[18]

However, little is known about the long-term effects of PTX on cytokine production and survival following thermal injury and subsequent septic challenge. So far most of the information on the therapeutic efficacy of PTX on sepsis was gained in short-term shock models where treatment was started immediately before or after the challenge and continued only for up to 36 hours.[19,20] The purpose of this study was to evaluate PTX and its effect on cytokine production in a mouse model of thermal injury and to study its effect on survival after septic challenge.

MATERIALS AND METHODS

Burn Injury

All procedures were carried out after review and with permission of the Harvard Medical School's Standing Committee on Animal Research using National Institutes of Health guidelines. As previously described,[3] 8-week-old male A/J mice (Jackson Labs, Bar Harbor, Maine) were randomized to receive either sham burn or burn injury. A standard 20% full-thickness scald burn to the dorsum was administered by inducing general anesthesia with pentobarbital (50 mg/kg diluted in about 0.75 mL sterile saline, intraperitoneally (IP)), shaving the dorsum of the mouse, placing the animal in a plastic template and immersing for 9 seconds in water at 90°C. Animals were resuscitated with 1 mL of saline IP after burn. Sham burned animals underwent the same procedure, but were immersed in thermoneutral water.

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Septic Challenge

At day 10 after burn injury septic challenge was administered by means of a standard cecal ligation and puncture technique (CLP). Under anesthesia, as above, the cecum was delivered through midline incision, ligated at its base, punctured twice with a 25-gauge needle, allowing expression of feces. The abdomen was closed and the animals resuscitated with 1 mL 0.9% saline. Animals were observed at least once daily and survival after septic challenge determined.

Therapeutic Intervention

Therapy with PTX (kindly provided by Hoechst, Somerville, New Jersey), recombinant human IL-2 (rhIL-2) (the kind gift of Amgen, Thousand Oaks, Calif.), or saline vehicle was given by intraperitoneal injection in a volume of 1 mL/d. rhIL-2 was diluted in 5% dextrose and PTX in saline.

Culture Media

Complete medium was RPMI-1640 containing 1% penicillin/streptomycin/fungizone (10,000 U/10,000 μg/25 μg/mL), 1 mmol HEPES, 5×10^{-5} M 2-mercaptoethanol and 2 mmol glutamine with addition of 5% heat-inactivated (56°C, 30 minutes) fetal calf serum (FCS). Serum-free medium contained all additives except FCS.

Cell Cultures

Mice were killed at day 4, 7, and 10 after burn injury, and splenocytes 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 microtiter flat-bottomed plates for 1 hour at 37°C in complete medium. Plates were washed three times to remove nonadherent cells and adherent cells were cultured in serum-free medium.

Interleukin-2 (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 microtiter plates in the presence of Con A at end well concentrations of 2.5 μg/mL at 37°C in 5% CO_2. After 48 hours supernatants were harvested, and frozen at −20°C for later IL-2 assay. For bioassay of IL-2, the supernatants were diluted from 1:2 to 1:128 in 100 μL of complete medium, and incubated for one hour at 37°C and 5% CO_2 to ensure proper pH. CTLL-2 cells, after appropriate washing 3 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 MTT. After 4 hours, cells were solubilized by the addition of 10% SDS, and uptake and conversion of MTT to formazan was determined in an automated ELISA reader (Molecular Devices, Mountain View, Calif.) 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 U/mL) using probit analysis (computer software kindly provided by Dr. Brian Davis of Immunex Corp., Seattle, Wash.).

Production of Monokines by Adherent Cells

Adherent cells obtained as above were cultured in the pre ence of *Escherichia coli* 026:B6 lipopolysaccharide (LPS (Sigma, St. Louis, Mo.) at an end well concentration of μg/mL. At 24 hours supernatants were removed and fro:en above until tested for monokine production.

Interleukin-6 (IL-6) Bioassay

Supernatants were tested for IL-6 using the IL-6–depende cell line B9 (generously provided by Genetics Institute, Can bridge, Mass. with kind permission of Dr. Lucien Aarde Netherlands Red Cross Transfusion Service). Supernatan were added to wells in serial dilutions from 1:2 to 1:128 an plates were equilibrated at 37°C and 5% CO_2 for 1 hour an then 5×10^3 appropriately washed B9 cells per well we added. Plates were incubated under the same conditions f 68 hours, followed by the addition of MTT, incubated ar read as above. IL-6 production in U/mL was then calculate from standard curves of recombinant IL-6 using probit ana ysis as for IL-2.

Tumor Necrosis Factor-Alpha (TNF-α) ELISA

TNFα was measured using a "sandwich" ELISA technique modified[4] from Sheehan et al.[21] Adherent cell supernatan were incubated in 96-well ELISA plates coated with hamst monoclonal anti-murine TNF-α antibody (Genzyme, Bosto Mass.). Plates were washed and incubated sequentially wi rabbit polyclonal anti-TNF-α antibody (Genzyme), and alk line phosphatase-conjugated goat anti-rabbit IgG (Boehring Mannheim, Indianapolis, Ind.). The alkaline phosphatas linked antibody was then detected in an automated imm nosorbent assay (ELISA) reader described above at 400 n after incubation with the phosphatase substrate *p*-nitrophen phosphate disodium (Sigma Chemical Co., St. Louis, Mo.

Prostaglandin E_2 (PGE_2) Assay

For the quantitative measurement of PGE_2 in supernatants LPS-stimulated adherent splenocytes a commercially ava able radioimmunoassay kit (Advanced Magnetics Inc., Ca bridge, Mass.) was used.

Statistical Analysis

Cytokine production and PGE_2 production were compar using the Mann-Whitney test for nonparametric data usi the statistics program INSTAT2. For survival data Kapl Meier survival analysis was used, with z and p values tained using the Wilcoxon statistic using the statistic progr Stata. Data are indicated as different if $p < 0.05$.

RESULTS

Effect of PTX Therapy on In Vitro Cytokine Secretion

Four groups of 30 mice each were treated for a total of 4, or 10 days (n = 10/group/d) beginning on the day 1 after bu or sham injury and total (IL-2) or adherent splenocyte (IL TNF, and PGE_2) secretion of cytokines determined as abo The four groups for each day were (a) sham burn injury.

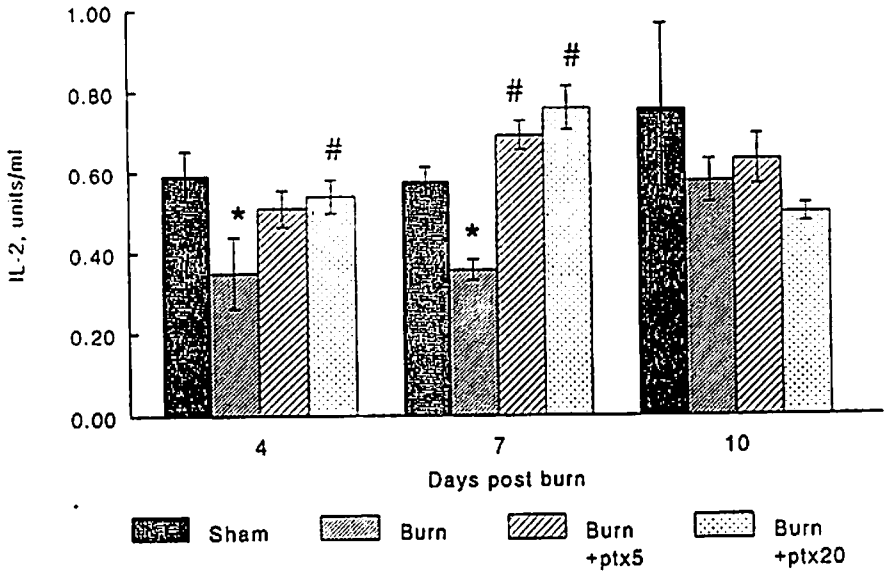

FIG 1. IL-2 production by mouse splenocytes at day 4, 7, and 10 after burn or sham injury. Burned mice were treated for days 1 through 4, 7, or 10 with either vehicle, PTX 5 mg or 20 mg/kg/d. Sham animals received saline. * = $p < 0.05$ compared to sham controls, # = $p < 0.01$ compared to burned + vehicle.

burn injury + vehicle control, (c) burn injury followed by therapy with PTX 20 mg/kg/d, and (d) burn injury with PTX 5 mg/d. Therapy was begun on day 1; i.e., the day after sham or burn injury. Sham animals received saline.

IL-2 deficiency (Fig. 1) in burned untreated mice was evident on day 4 (burn 0.35 + 0.09 U/mL, sham, 0.59 + 0.06 mean +/− SEM; $p < 0.05$) and on day 7 (burn 0.36 + 0.03 U/mL, sham 0.58 + 0.04; $p < 0.001$). PTX 20 significantly improved IL-2 production in splenocytes on day 4 (0.54 + 0.04 U/mL $p < 0.05$ compared with burn) and on day 7 both PTX5 and PTX 20 restored IL-2 production by splenocytes from burned mice to levels significantly higher than burned mice ($p < 0.001$) and not different from sham controls ($p > 0.05$). On day 10 there was no significant difference in IL-2 production among any of the groups.

On day 4 following injury there was a marked deficiency of IL-6 secretion by LPS-stimulated adherent splenocytes in all groups, suggesting that this may be an effect of anesthesia and sham burn (Fig. 2). On day 7 PTX 20 caused a significant decrease in IL-6 production (0.4 U/mL; $p < 0.01$) compared to the burned untreated group (median 8.6 U/mL). Neither treatment affected IL-6 secretion on day 10.

TNFα production was significantly increased on day 7 in the burned untreated group (mean 2426.8 pg/mL; $p < 0.001$) compared with results from sham treated mice, but was not different on either day 4 or day 10. On day 7 both PTX 5 mg/kg ($p < 0.01$) and PTX 20 mg/kg ($p < 0.01$) decreased TNF-α production. On day 10 there was only a marginally significant ($p = 0.1797$) difference in the PTX 20 group (Fig. 3).

PGE_2 production by LPS-stimulated adherent splenocytes was significantly elevated in all burned mice with vehicle saline injection on day 4, 7, and 10 with a maximum on day 4. The adherent cells from mice treated with PTX 20 mg/kg had a significantly lower PGE_2 production on day 4, 7, and 10 (all $p < 0.001$) and those from mice treated with PTX5 were significantly decreased on day 7 compared to vehicle-treated burned mice (Fig. 4).

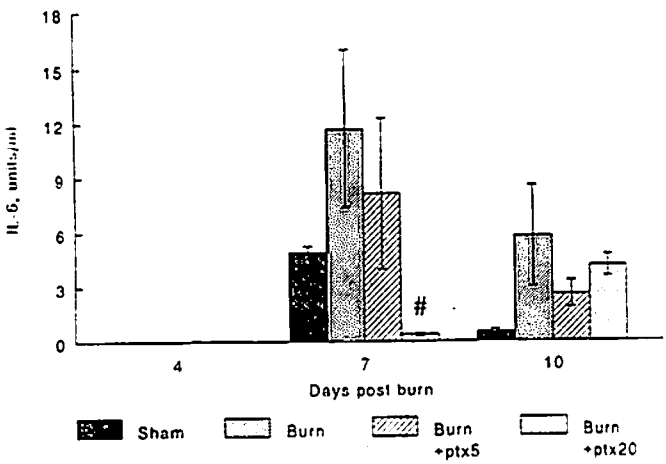

FIG 2. IL-6 production at day 4, 7, and 10 after burn injury by mouse adherent splenocytes. Groups as in Figure 1.

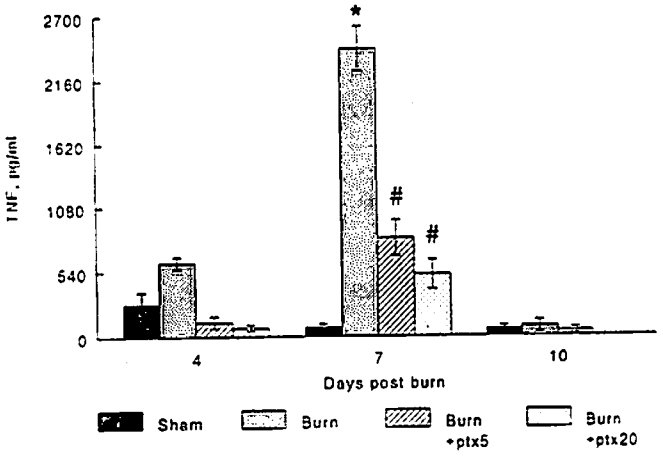

FIG 3. TNF production at day 4, 7, and 10 after burn injury in mouse splenocytes. Groups as in Figure 1.

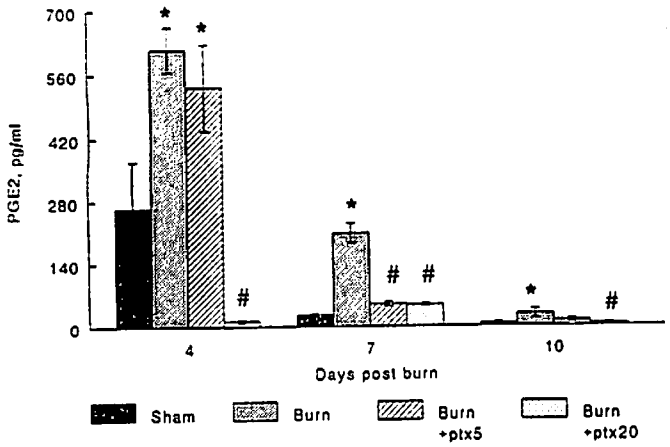

FIG 4. PGE_2 production at day 4, 7, and 10 after burn injury in mouse splenocytes. Groups as in Figure 1.

Survival Experiments

In the first study the effect on survival following burn/CLP of PTX (5 mg/kg/day; n = 10), rhIL-2 (250 U/mouse/d; n = 10) a combination of PTX and rhIL-2 (n = 10) at the same doses, or saline vehicle (n = 10), was tested. Mice received a burn injury and a CLP at day 10 after burn injury. The burned animals were randomized into groups carefully matched for weight to receive intraperitoneal injections as described starting on the day 1 after burn and continuing for a maximum of 10 days after the septic challenge. In the second study, the treatment was started at the day of CLP with PTX (n = 10), rhIL-2/PTX (n = 9), or saline vehicle (n = 10), and continued for 10 days.

In the first survival study, in which treatment was started immediately after burn injury, PTX treatment alone did not improve the survival rate, but a combination of low-dose IL-2 and PTX ($p < 0.05$) was superior to single treatment with either IL-2 or PTX (Fig. 5). When treatment was given starting only beginning on the day of CLP (i.e., day 10 after burn) PTX improved the survival rate ($p < 0.05$), but a combination of IL-2 and PTX did not (Fig. 6).

DISCUSSION

Previous studies using this mouse model have shown that thermal injury is followed by a period of increasing impairment in T-lymphocyte activation and IL-2 production and the

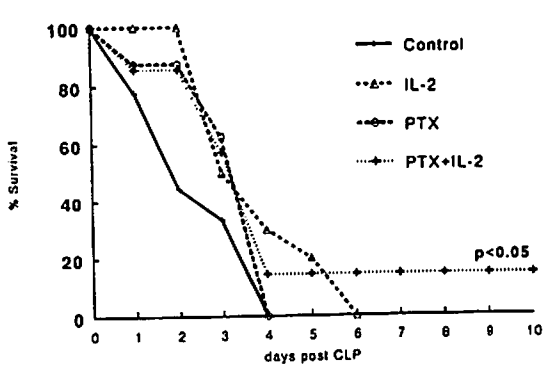

FIG 5. Survival after burn injury and CLP therapy started at burn injury.

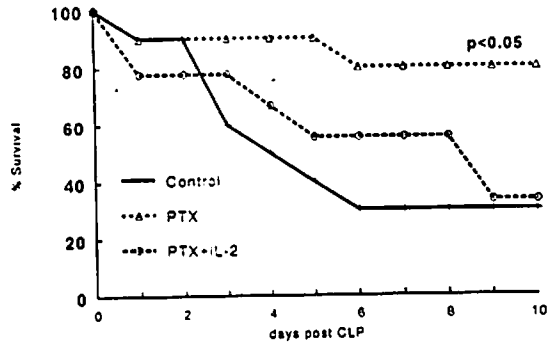

FIG 6. Survival after burn injury and CLP. Therapy started on day of CLP.

time of peak impairment of these lymphocyte functions correlates with the peak mortality from a septic challenge from CLP.[2,3] Furthermore, secretion of TNF-α and IL-6 by adherent cells in the splenocyte population from burned mice was found to be markedly increased compared to controls.[4]

Similarly, in the present study, in the burned untreated mice we found a simultaneous decrease in IL-2 production by lymphocytes from burned mice on day 4 and 7 after injury in the untreated group as well as elevation of macrophage PGE_2, TNF-α, and IL-6 production. The suppression of IL-2 production correlated with elevated PGE_2 production on day 4, and the moderately elevated production on day 7 might be sufficient to suppress IL-2 production. Lymphocytes in burn patients are in fact more sensitive to the suppression exerted by PGE_2,[22] and PGE_2 has previously been related to the suppression of IL-2 production in this mouse model.[23] It seems likely therefore that PTX elevated IL-2 production by suppression of PGE_2 secretion.

Whereas PGE_2 had already peaked on day 4, TNF-α production reached its maximum on day 7 when PGE_2 production was only moderately elevated in the untreated group. At this moderately elevated level PGE_2 might actually be stimulatory for macrophages, as was observed by Renz et al.[24] whereas high levels might suppress macrophage TNF-α production on day 4. The increased cytokine production of macrophages (TNF, IL-6) might also be due to a loss of sensitivity of macrophages to the inhibitory effects of PGE_2.[25,26]

Pretreatment of macrophages with indomethacin, which inhibits PGE_2 metabolism in vitro, was associated with an enhancement of PGE_2-induced suppression of TNF-production.[25] A restoration of this sensitivity in the macrophage by indomethacin may lead to a recovery of macrophage control and downregulation of cytokine production.

IL-6, a cytokine whose synthesis and secretion can be induced by TNF, was also downregulated by PTX on day 7 in our study. This could be due to a significantly reduced TNF production at that time. In contrast, in human volunteers who received endotoxin infusion IL-6 production did not differ among the PTX treatment group, untreated or controls.[27] This may be due to the fact that IL-6 measurements were measured only early after endotoxin whereas we have demonstrated in the mouse model that IL-6 is released later in the time course than TNF or PGE_2. Most of this takes place in our model after day 7.[4] In rats high doses of PTX may cause a rise in plasma

IL-6 levels, but not in TNF, whereas pretreatment with PTX blunts the peak level of IL-6 and TNF in rats following injection of LPS.[28]

The overall effect of prolonged macrophage hyperactivity appears to be detrimental. Downregulation of the macrophage cytokine production by PTX should then be associated with an increase in survival after the septic challenge in our model. We could demonstrate that a combination therapy of low dose IL-2, which is ineffective by itself, and PTX started after thermal injury and continued after septic challenge, was able to improve the survival rate significantly, although only moderately. PTX alone showed no effect. Macrophage blockade might be associated with increased mortality from sepsis in normal mice.[29] Because from the previous study we found that PTX (20 mg/kg) decreased TNF production on day 10, we hypothesized that this relatively moderate improvement in survival rate might be due to this downregulating effect of PTX at day 10. In a second experiment we started treatment after septic challenge on day 10 and could demonstrate a significantly increased survival rate after PTX treatment alone.

In the standard animal burn without infectious challenge mortality is usually <10%.

The survival rate in the first experiment was calculated by Kaplan-Meier survival analysis of the survival curve, and not a single endpoint, using the Wilcoxon statistic. Despite statistical significance this result is probably not of clinical importance. The improvement in the survival rate became obvious only in the second survival experiment, when PTX treatment was started after the induction of sepsis. In this experiment the saline-treated mice showed a different mortality compared to the survival rate of the saline-treated mice in the first experiment. There is certainly an effect of the daily injections beginning immediately after the burn. It is known that mice react even to the stress of being anesthetized, shaven and immersed once in room temperature water with a significant immune suppression. The variation also can be explained by physiological differences between one batch of mice and another. Also in this model there is likely to be experimental variation. The two survival experiments were carried out at different times and conditions may not have been exactly the same; e.g., although mice are supposed to be 7 to 8 weeks of age there may be more age variation from one batch to the next.

The clinical significance of our findings is that we can use PTX in the treatment of the inflammatory response during the days after injury and even after sepsis occurred. Because it is difficult to predict the exact time when to start therapy as it is necessary with monoclonal antibodies against TNF and endotoxin, the use of PTX expands the window of time in which therapy could be successful.

Although PTX was able to restore T-cell production of IL-2 and downregulated proinflammatory cytokines (e.g., TNF) it still did not improve survival to 100%. TNF or IL-2 seem not to be the only mediators influencing the outcome after septic challenge.

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