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The regulation of burn-associated infections with herpes simplex virus type 1 or *Candida albicans* by a non-toxic aconitine-hydrolysate, benzoylmesaconine. Part 1: Antiviral and anti-fungal activities in thermally injured mice

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Summary As compared with normal unburned mice, thermally injured mice have been shown to be 50–100 times more susceptible to HSV type 1 (HSV-1) or *Candida albicans* infection. Benzoylmesaconine (BEN) improved the resistance of thermally injured mice against infection with HSV-1 or *C. albicans* to the level observed in normal mice. Mortality rates of normal mice exposed to lethal amounts of these pathogens were not affected by the BEN treatment, while significant survival effects were produced in these mice after treatment with acyclovir (against HSV-1) or amphotericin B (against *C. albicans*). Benzoylmesaconine did not inhibit the growth of these pathogens *in vitro* and did not directly reduce the viability of the pathogens. However, burned mice inoculated with $CD4^+$ T cells from BEN-treated mice resisted infections from these pathogens. These results suggested that, through the generation of $CD4^+$ T cells, BEN recovered the impaired resistance of thermally injured mice to infection by HSV-1 or *C. albicans*.

Key words: benzoylmesaconine, *Candida albicans*, HSV, thermal injury.

Introduction

Between 50 and 80% of the mortality of thermally injured patients is due to infections.[1–5] Both clinical and laboratory studies have shown that there is an increased susceptibility of thermally injured patients to infection, over and above that associated with the loss of the skin barrier.[3–5] Certain therapies currently available against bacterial infections include antibiotics, cytokines and mAbs directed against TNF-α or endotoxins.[6–8] However, viral and fungal infections in thermally injured patients still remain difficult to treat.[9] Herpesviruses (HSV and cytomegalovirus) and *Candida albicans* have been reported as severe pathogens in thermally injured patients,[3–5] although these organisms express minimal pathogenic effects in healthy individuals. Infections with herpesviruses are of special concern because they are not only significant pathogens themselves, but they also compound the concern of infection by making the patient more susceptible to other pathogens.[3] In a previous report,[10] although only 21% of thermally injured patients developed invasive *Candida* sepsis, over 90% of these patients died.

The major underlying reasons for the increased susceptibility of burned patients to these infections are immunological abnormalities associated with thermal injuries.[11–13] In our animal studies,[14,15] the susceptibility of burned mice to infections by HSV-1 or *C. albicans* was 50–100 times greater than that of normal mice. The increased susceptibility of burned mice to these infections was correlated with the generation of burn-associated $CD8^+$ $CD11b^+$ $TCR\gamma/\delta^+$ type-2 T cells.[14] The increased susceptibility of thermally injured mice to these pathogens has been completely transferred to normal unburned mice by the adoptive transfer of burn-associated type-2 T cells.[14] In addition, the impaired resistance of thermally injured mice to these pathogens was restored to the levels obseved in normal mice, when type-2 T cell responses were blocked in thermally injured mice through the treatment of anti-type-2 cytokine mAbs.[15] These facts suggest that type-2 T cell responses associated with burn injury are major factors on the impaired resistance of thermally injured mice to infections with these pathogens.

We have previously demonstrated the survival of thermally injured mice exposed to lethal amounts of HSV-1 when Kanzo-bushi-to, a traditional Chinese herbal medicine, was administered to these mice.[16] Benzoylmesaconine (16-methyl-1, 6, 19-trimethoxy-4-(methoxymethyl)aconitane-3, 8, 11, 18-pentol 10-benzoate) is a crystallized aconitine-hydrolysate purified from heated *Aconiti* tuber,[17] which is an active constituent of Kanzo-bushi-to against HSV-1 infection.[16] Although an alkaloid aconitine contained in the *Aconitum* species is a powerful poison,[18] BEN (the product of a hydrolysed aconitine) only expresses a very weak toxic effect.[19] Therefore, in the present study, the effects of BEN on the impaired resistance of burned mice

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exposed to HSV-1 or *C. albicans* were investigated. The results obtained indicated that BEN restored the impaired resistance of burned mice to HSV-1 and *C. albicans* infections through the induction of effector $CD4^+$ T cells.

Materials and methods

Animals

Eight-week-old BALB/c mice (The Jackson Laboratories, Bar Harbor, ME, USA) were used in the experiments. All procedures involving animal experiments were approved by the Animal Care and Use Committee of the University of Texas Medical Branch at Galveston (ACUC approval number: 94–06–028).

Thermal injury

As described previously,[20] all animals (24–26 g) were anaesthetized by pentobarbital (40 mg/kg, i.p.). Animals were shaved over the dorsum and placed under a specially constructed asbestos cloth with a window uncovering 15% of their total body surface area. Then, they were exposed for 9 s to a gas flame using a Bunsen burner equipped with a flame dispersing cap. This procedure resulted in a third degree burn in mice over ~15% total body surface area.[21] Animals were immediately resuscitated i.p. with 4 mL of saline per mouse.

Cells, media, HSV-1 and Candida albicans

Vero cells were maintained serially in MEM supplemented with 10% foetal bovine serum, 2 mmol/L L-glutamine and antibiotics. The KOS strain of HSV-1 was propagated in Vero cells and stored at −70°C until used for inoculation.[14,15] The titre of the virus stock solution was 1.8×10^7 p.f.u./mL, as assayed by the plaque method on Vero cells cultured in maintenance medium (MEM supplemented with 2% foetal bovine serum, 2 mmol/L L-glutamine, penicillin and streptomycin).[14,15] A wild strain of *C. albicans*, isolated from a patient at the University of Texas Medical Branch, Galveston, Texas, USA was serially maintained on agar plates of Sabouraud dextrose medium.

BEN

Benzoylmesaconine (BEN), a hydrolysed aconitine purified from methanol extracts of heated *Aconiti* tuber,[17] was supplied by Tsumura Central Research Institute (Ibaraki, Japan). Benzoylmesaconine was dissolved in saline at a concentration of 1 mg/mL, then diluted to the appropriate concentrations by the medium or saline when it was used in experiments. This preparation was orally administered to mice by a 20 G feeding needle. According to the preliminary studies related to Kanzo-bushi-to,[16] BEN was administered to mice 2 days before, and 1 and 3 days after the infection.

Infection experiments

One day after thermal injury mice were infected with appropriate amounts of HSV-1 (i.p.) or *C. albicans* (i.v.). An inoculum of 1×10^4 p.f.u./mouse in normal mice and 1×10^2 p.f.u./mouse in burned mice have previously been determined to be equivalent to 1 LD_{50} of HSV-1.[14] An inoculum of 1×10^7 cells/mouse in normal mice and 2×10^5 cells/mouse in burned mice was shown to be equivalent to 1 LD_{50} of *C. albicans*.[21] This indicates that the susceptibility of burned mice to HSV-1 or *C. albicans* infections was 50 (*C. albicans*)–100 (HSV-1) times greater than that in normal mice. Acyclovir (Burroughs Wellcome Co., NC, USA) and amphotericin B (Sigma Chemical Co., St Louis, MO, USA) were used as positive controls for HSV-1 (acyclovir) and *C. albicans* (amphotericin B).[23,24] Mice exposed to various pathogens were treated twice a day with various doses of acyclovir (1–40 mg/kg, orally) or amphotericin B (1 mg/kg, i.p.) for a total of 7 days beginning 1 day after the infection.[23,24] To determine the growth of *C. albicans* in mice, kidneys removed from the infected mice were disrupted with a glass homogenizer in combination with sonication.[25] A 10% suspension of these homogenates in physiological saline was assayed for fungal titres in Sabouraud dextrose agar. As described previously,[15] the resistance of burned mice to the infection was evaluated using the following criteria: (i) the mean survival time in days (MSD) of tested groups as compared with MSD of control mice treated with saline; and (ii) the survival percentage of tested groups 3 weeks (HSV-1) or 4 weeks (*C. albicans*) after the infection as compared with that of controls. All of the experiments were performed two times and the results shown were displayed by mean values of the two experiments.

Adoptive transfer

Mononuclear cells (SMNC) were prepared by Ficoll-Hypaque sedimentation from spleens of normal or burned mice treated with BEN, as described previously.[14,15] SMNC from normal or burned mice treated with saline were used as control cells. To obtain purified whole T cells, $CD4^+$ T cells or $CD8^+$ T cells, SMNC (5×10^7 cells/mL) were applied to T cell columns (whole T cells), CD4 subset columns ($CD4^+$ T cells) or CD8 subset columns ($CD8^+$ T cells; R&D Systems, Minneapolis, MN, USA).[14,15] When whole T cells were treated with anti-Ig antiserum and complement, only a 3% reduction in viable cells was demonstrated, whereas treatment of these cells with anti-CD3 mAb followed by complement caused a 98% reduction in the number of viable cells. When $CD4^+$ T cell fractions or $CD8^+$ T cell fractions were treated with anti-L3T4 mAb followed by complement, 96% or 3% of viable cells were lysed, respectively. When they were treated with anti-Lyt 2.2 mAb followed by complement, 2% or 97% of viable cells were lysed, respectively. These results indicated that the purity of these three cell preparations (whole T cells, $CD4^+$ T cells and $CD8^+$ T cells) was more than 96%. One day after the thermal injury, mice were inoculated i.v. with 2×10^7 cells/mouse of SMNC, 1×10^7 cells/mouse of whole T cells, 5×10^6 cells/mouse of $CD4^+$ T cells or 5×10^6 cells/mouse of $CD8^+$ T cells obtained through the procedures. Two hours after the inoculation, these mice were exposed to a 5 LD_{50} dose of HSV-1 (5×10^2 p.f.u./mouse) or *C. albicans* (1×10^6 cells/mouse). The resistance of recipient mice to the infection was as described earlier.

Statistical analyses

Results were analysed statistically using Student's *t*-test (MSD) and χ^2 analysis (% survival of mice exposed to pathogens). If a P value was lower than 0.05, the result obtained was considered significant.

Results

BEN improves resistance of thermally injured mice exposed to HSV-1

In comparison with normal mice, burned mice are 100 times more susceptible to HSV-1 infection.[14,15] Using thermally

injured mice, the effect of BEN on the susceptibility to HSV-1 infection was examined (Fig. 1). When burned mice were exposed to 5×10^2 p.f.u./mouse of HSV-1 (5 LD_{50} in burned mice) and treated with BEN, 80% of these mice survived more than 3 weeks after the infection ($P<0.001$, χ^2 analysis). In contrast, after the infection with the same amount of HSV-1, 5% of burned mice treated with saline and 40% of burned mice treated with a 10 mg/kg dose of acyclovir survived (Fig. 1). The dose-dependent protective effect of BEN on burned mice to HSV-1 infection is shown in Fig. 2. The highest protection (80% survival) of burned mice exposed to 5 LD_{50} of HSV-1 (5×10^2 p.f.u./mouse) was revealed when the mice were treated with BEN at a dose of 1 μg/kg (Fig. 2a). However, these protective effects of BEN against the infection of HSV-1 were not observed in normal unburned mice (Fig. 2b). All of normal mice treated with BEN at various doses ranging from 0.01 μg/kg to 10 mg/kg died after the infection of 5 LD_{50} of HSV-1 (5×10^4 p.f.u./mouse). In contrast, acyclovir produced survival effects in both groups of mice exposed to appropriate doses of HSV-1 (Fig. 2a,b). These results suggest that BEN protects thermally injured mice infected with HSV-1 through the regulation of burn-affected host functions.

Effect of BEN on C. albicans *infection in burned mice*

Similar protective effects of BEN were shown in thermally injured mice exposed to *C. albicans* (Table 1). An inoculation of 1×10^6 organisms/mouse of *C. albicans* produced a 100% mortality in burned mice and a 0% mortality in normal mice (Table 1, Expt 2). The number of *C. albicans* required for 5 LD_{50} was 5×10^7 cells/mouse in normal mice and 1×10^6 cells/mouse in burned mice (Table 1, Expt 1 and 2). As compared with normal mice, therefore, burned mice were shown to have 50-fold greater susceptibility to *C. albicans* infection.[22] All of the burned mice exposed to 5 LD_{50} of *C. albicans* (1×10^6 cells/mouse) survived when they were treated with a 1 μg/kg dose of Benzoyl (Table 1, Expt 3). However, the same dose of the compound had no protective effect in normal mice infected with 5 LD_{50} of the *C. albicans* (5×10^7 cells/mouse; Table 1, Expt 1). Amphotericin B used as a positive control produced a 50% survival rate in normal mice and a 25% survival rate in burned mice exposed to respective 5 LD_{50} of *C. albicans* (Table 1, Expt 1 and 3). The growth of *C. albicans* was determined in kidneys of normal mice, burned mice and burned mice treated with BEN (Fig. 3). When normal mice were infected with 1×10^6 cells/mouse of *C. albicans* (which corresponds to 0.05 LD_{50} in normal mice), 8.1×10^4 cells/organ of the organisms were detected in kidneys of these mice 7 days after the infection. However, *C. albicans* in kidneys of normal mice was reduced to 3.0×10^3 organisms/organ 2 weeks after the infection (Fig. 3a). As compared with normal mice, the increased fungal load in kidneys (2.9×10^6 and 8.8×10^7 organisms/organ 7 and 14 days after the infection,

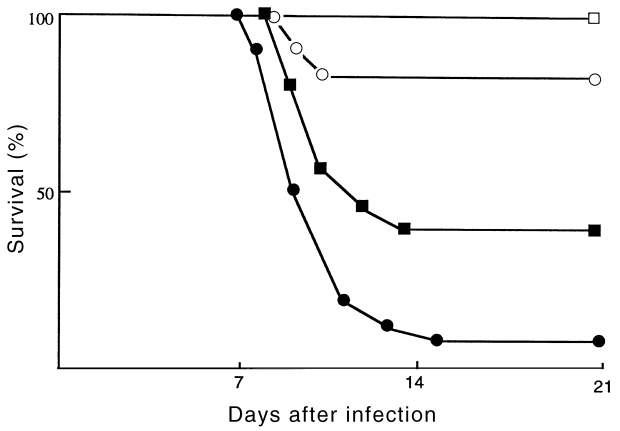

Figure 1 Benzoylmesaconine (BEN) improved the resistance of thermally injured mice exposed to HSV-1. Mice 1 day after thermal injury (○, ●, ■) or normal mice (□) were infected with HSV-1 at a dose of 5×10^2 p.f.u./mouse (corresponded to 5 LD_{50} in burned mice and 0.05 LD_{50} in normal mice). Benzoylmasaconine (1 μg/kg) was administered orally to burned mice 2 days before, 1 and 3 days after the infection (○). Normal (□) and burned mice (●) treated with saline (0.2 mL/mouse, orally) served as controls. As a positive control, burned mice exposed to HSV-1 were treated with acyclovir (10 mg/kg, orally, twice daily for a total of 7 days) beginning 1 day after the infection, (■). The results illustrated in the figure were obtained from 10 mice/group in two separate experiments.

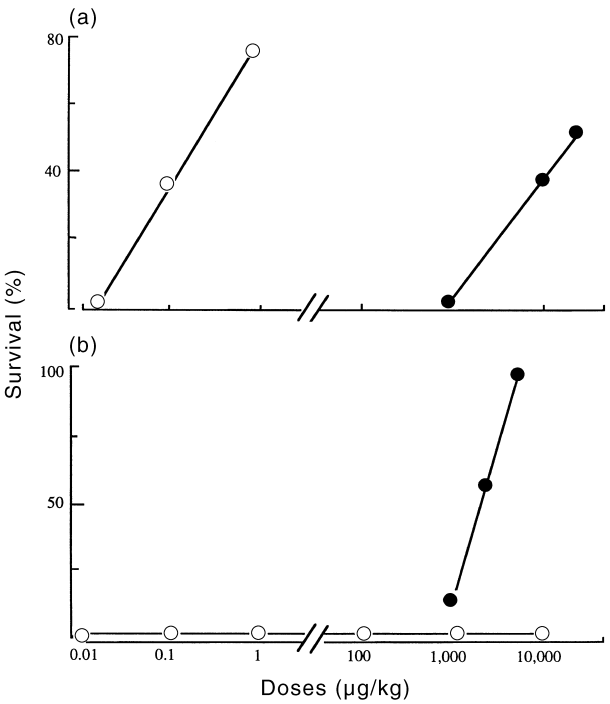

Figure 2 Dose–response effect of benzoylmesaconine (BEN) on the survival of burned mice infected with HSV-1. (a) Mice 1 day after thermal injury or (b) normal mice were infected with 5 LD_{50} of HSV-1 ((a) 5×10^2 p.f.u./mouse; (b) 5×10^4 p.f.u./mouse). These mice were treated orally with various doses of BEN 2 days before, 1 and 3 days after the infection (○). As controls (●), normal and burned mice exposed to HSV-1 were treated with various doses of acyclovir (twice daily for a total of 7 days beginning 1 day after the infection). The data present the survival percentage from eight mice/group in two separate experiments.

respectively) was observed in burned mice infected with 1×10^6 cells/mouse of *C. albicans* (which corresponds to 5 LD_{50} in burned mice; Fig. 3b). When burned mice were exposed to the same amount of *C. albican*s and treated with BEN, 3.3×10^4 and 3.2×10^3 organisms/organ of fungal cells were detected in mice 7 and 14 days after the infection, respectively, (Fig. 3). These results suggest that the resistance of burned mice to the *C. albicans* infection was improved after the treatment with BEN.

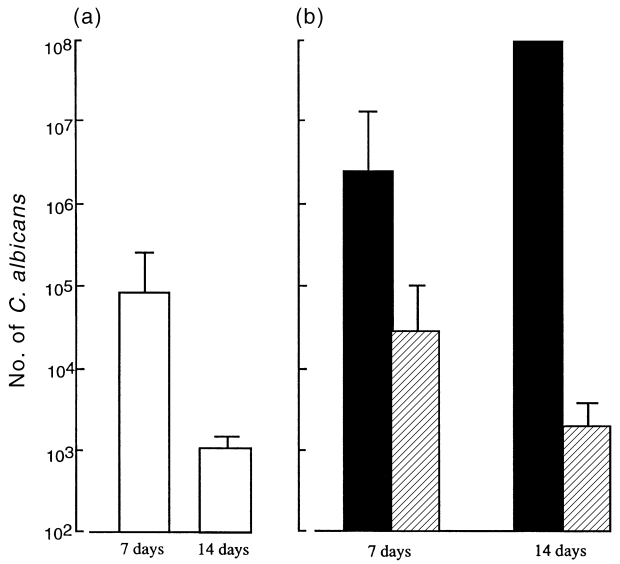

Figure 3 The growth of *Candida albicans* in kidneys of burned mice. Burned mice were treated with benzoylmesaconine (BEN) 2 days before, 1 and 3 days after infection of *Candida albicans* (1×10^6 cells/mouse, 5 LD_{50} in burned mice). As a control, normal mice exposed to the same amounts of *Candida albicans* were treated orally with saline (0.2 mL/mouse). These mice were killed 7 and 14 days after the infection, and the numbers of viable fungal cells in kidneys were measured, as described in the Materials and methods. The results expressed are the mean ± SD of triplicate determinations. (□), normal mice; (■), burned mice treated with saline; (▨), burned mice treated with BEN.

Effect of BEN on the growth or viability of pathogens in vitro

When HSV-1 or *C. albicans* were grown in Vero cells (HSV-1)[26] or in Sabouraud dextrose medium (*C. albicans*)[25] in the presence of BEN (0.01–100 μg/mL), their replications were not altered. Thus, BEN has no inhibitory activities on the growth of these pathogens *in vitro*. A 1×10^5 p.f.u./mL of HSV-1 or a 1×10^6 cells/mL of *C. albicans* was mixed with BEN at concentrations ranging from 0.1–100 μg/mL and kept at 37°C for 24 h. Then, residual titres of the pathogens were determined in cultures of Vero cells (HSV-1), or on the agar plates of Sabouraud dextrose medium (*C. albicans*). However, the viabilities of these pathogens were not affected by BEN directly (data not shown). These results indicate that host's functions against pathogens are required when thermally injured mice exposed to the pathogens are protected by BEN. Therefore, in the next experiment, various effector cells from BEN-treated mice were adoptively transferred to burned mice exposed to the pathogens.

Effect of adoptively transferred splenic T cells from BEN-treated mice on the resistance of burned mice to infection with HSV-1 or C. albicans

Whole T cells, $CD4^+$ T cells and $CD8^+$ T cells were prepared from SMNC of normal mice treated with BEN. Then,

Table 1 Protective effect of benzoylmesaconine (BEN) on *Candida albicans* infection in burned mice

Mouse	Inoculum size (cells/mouse)	LD_{50}	Treatment and dose	MSD (days)	Survived/treated (%)
Expt 1					
Normal	5×10^7	5	Saline, 0.2 mL/mouse	> 12.2	1/20 (5)
Normal	5×10^7	5	BEN, 1 μg/kg	10.4	0/20 (0)
Normal	5×10^7	5	Amphotericin B, 1 mg/kg	> 22.0***	10/20 (50)**
Expt 2					
Normal	1×10^6	0.1	Saline, 0.2 mL/mouse	> 28.0	10/10 (100)
Burn	1×10^6	5	Saline, 0.2 mL/mouse	9.7	0/10 (0)
Expt 3					
Burn	1×10^6	5	Saline, 0.2 mL/mouse	> 10.5	1/20 (5)
Burn	1×10^6	5	BEN, 1 μg/kg	> 28.0***	20/20 (100)***
Burn	1×10^6	5	BEN, 0.1 μg/kg	> 21.8***	10/20 (50)**
Burn	1×10^6	5	BEN, 0.01 μg/kg	10.2	0/20 (0)
Burn	1×10^6	5	Amphotericin B, 1 mg/kg	> 15.0*	5/20 (25)

Groups of 20 normal mice or mice 1 day after thermal injury were infected with *C. albicans* and treated orally with various doses of BEN 2 days before and 1 and 3 days after the infection. Groups of 10 to 20 mice treated orally with saline (0.2 mL/mouse) were exposed to *C. albicans* and served as controls. As positive controls, normal and burned mice exposed to *C. albicans* were treated with amphotericin B. Amphotericin B was administered i.p. to mice twice daily for a total of 7 days beginning 1 day after the infection.[23] Mice in each group were observed daily for 4 weeks to determine the mean survival time in days (MSD) and survival rates. Statistical analysis; Student's *t*-test for MSD; χ^2 analysis for survival rates; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

these cells were adoptively transferred to burned mice previously infected with 5 LD_{50} of HSV-1 or *C. albicans*. SMNC derived from burned mice treated with BEN were also transferred to these recipient mice. The results obtained are shown in Fig. 4. After the infection of HSV-1 at a dose of 5×10^2 p.f.u./mouse, 95–100% of burned mice inoculated with naive T cells from normal mice or $CD8^+$ T cells from BEN-treated mice died. However, when burned mice received whole T cells and $CD4^+$ T cells from BEN-treated mice or SMNC from burned mice treated with BEN and exposed to 5 LD_{50} of HSV-1, 75–95% of them survived (Fig. 4a). After the infection with *C. albicans*, similar results were obtained in burned mice inoculated with various splenic cell populations from normal and burned mice treated with or without BEN (Fig. 4b). These results suggest that the impaired resistance of thermally injured mice to infections with HSV-1 and *C. albicans* was restored by the $CD4^+$ T cells derived from spleens of BEN-treated mice.

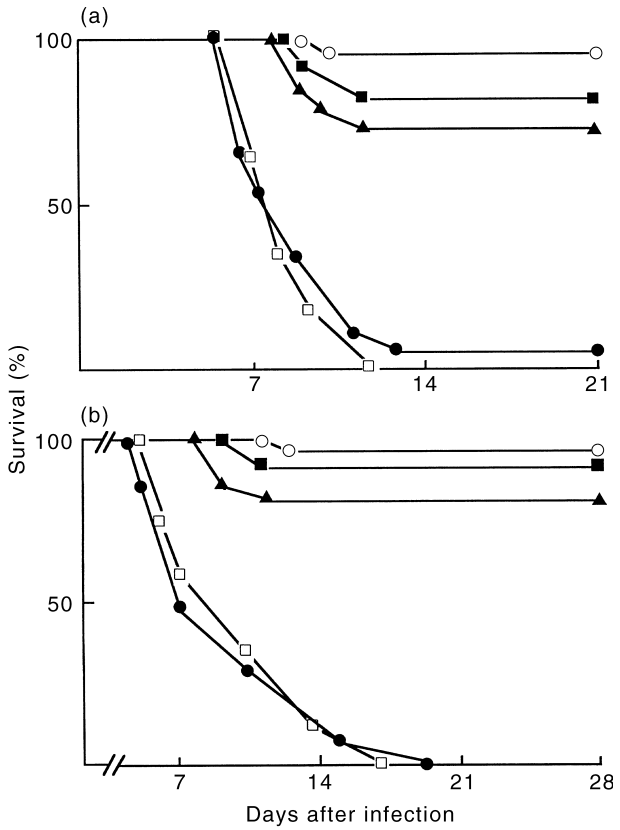

Figure 4 Effect of adoptively transferred splenic cells from benzoylmesaconine (BEN)-treated mice on the mortality of burned mice exposed to HSV-1 or *Candida albicans*. Mice 1 day after thermal injury were inoculated with whole T cells (1×10^7 cells/mouse, ■), $CD4^+$ T cells (5×10^6 cells/mouse, ○) or $CD8^+$ T cells (5×10^6 cells/mouse, □) from spleens of normal mice treated orally with BEN (1 μg/kg) 2 days before death. SMNC (2×10^7 cells/mouse, ▲) from burned mice treated with BEN were also adoptively transferred to burned mice. These mice were then infected with HSV-1 (a, 5×10^2 p.f.u./mouse) or *Candida albicans* (b, 1×10^6 cells/mouse) 2 h after the inoculation. As a control, burned mice inoculated with splenic T cells from normal mice (●) were infected with the same amount of pathogens.

Discussion

In our previous human studies,[27] a markedly decreased production of type-1 cytokines and the impaired IL-2-dependent proliferative responses were demonstrated in peripheral blood T cells from thermally injured patients. Also, the production of type-2 cytokines and IL-4-required proliferative responses of these cell preparations were demonstrated.[27] $CD3^+$ $CD8^+$ $CD30^+$ cells were identified as burn-associated type-2 cells in PBL of thermally injured patients.[27] CD30 antigen has been shown to be expressed in Th2 cells.[28–30] SCID mice reconstituted with PBL from thermally injured patients (patient PBL-SCID chimeras) were susceptible to the *C. albicans* infection, while SCID chimeras inoculated with PBL from healthy volunteers were resistant.[27] In addition, the impaired resistance of patient PBL-SCID chimeras to the infection was completely restored after the inoculation of $CD30^+$ cell-depleted PBL from thermally injured patients.[27] As patient PBL-SCID chimeras express an immune response similar to burned patients,[31] these results suggest that burn-associated $CD30^+$ type-2 T cells play a role in the increased susceptibility of thermally injured patients to the *C. albicans* infection.

In our previous animal studies,[14,15,22] the markedly increased susceptibility of thermally injured mice to infections with HSV-1 and *C. albicans* was demonstrated. In mice 3–9 days after thermal injury, type-1 cytokines were not produced even though they were stimulated with staphylococcal enterotoxin A or Con A.[20] As burn-associated type-2 T cells, $CD8^+$ $CD11b^+$ $TCR\gamma/\delta^+$ T cells were demonstrated in spleens of thermally injured mice.[14] Normal mice inoculated with burn-associated type-2 T cells became susceptible to infections from HSV-1 and *C. albicans* at the same levels shown in thermally injured mice.[14,22] Further, the increased susceptibility of thermally injured mice or normal mice inoculated with burn-associated type-2 T cells to infections was recovered to the levels observed in normal mice when they were treated with a mixture of mAbs against type-2 cytokines.[15] All of these facts indicate that burn-associated type-2 T cells and/or their type-2 cytokine products are a key in the increased susceptibility of thermally injured mice to the infections.

Anti-type-2 T cells, generated in spleens of thermally injured mice following the appearance of burn-associated type-2 T cells, have previously been demonstrated.[14] The suppressor cell activity of burn-associated type-2 T cells was determined by a mixed lymphocyte reaction and it was effectively inhibited by the anti-type-2 T cells *in vivo* and *in vitro*.[14] Also, the resistances of thermally injured mice to HSV-1 infections were completely improved when thermally injured mice were inoculated with burn-induced anti-type-2 T cells.[14] These anti-type-2 T cells were characterized as a *Vicia villosa* lectin-adherent $CD4^+$ $CD28^+$ $TCR\alpha/\beta^+$ T cell.[14] The phenotypic and functional properties of anti-type-2 T cells were shown to be similar to those of the

contrasuppressor T cells which have been previously reported.[32,33] Kupper and Green demonstrated that the contrasuppressor cells were involved in the recovery of patients from immunosuppression induced by thermal injury.[34] The anti-type-2 T cell-inducing activity of glycyrrhizin (an active component of licorice roots)[35,36] and Kanzo-bushi-to (a traditional Chinese herbal medicine)[16] in normal or burned mice has been previously demonstrated in this laboratory.

In the present study, the mortality rate of burned mice exposed to lethal amounts of HSV-1 or *C. albicans* was greatly reduced after the treatment with BEN. The maximum efficacy of BEN was observed when burned mice exposed to the pathogens were treated orally with a 1 μg/kg dose of the compound. As an 860 mg/kg oral dose of BEN had been shown to be 1 LD_{50} in mice,[19] the therapeutic index of the agent in this case was calculated to be $8.6 \times 10.^5$ The viability of HSV-1 and *C. albicans* was not reduced when the pathogens were incubated with BEN (0.01–100 μg/mL) for 24 h at 37°C. Also, the growth of HSV-1 was not inhibited in Vero cells treated with BEN. *Candida albicans* grew in Sabouraud dextrose medium supplemented with 0.01–100 μg/mL of BEN. These results suggested that BEN may express its activity through the regulation of the host's functions. When whole T cells or $CD4^+$ T cells, prepared from spleens of mice treated with BEN (donors), were adoptively transferred to burned mice (recipients) infected with HSV-1 or *C. albicans*, the impaired resistance of recipient mice to the infection was restored. However, the mortality rates of recipient mice stayed at 100% when they were inoculated with $CD8^+$ T cells from BEN-treated mice. When $CD4^+$ T cells from BEN-treated mice were adoptively transferred to normal mice infected with a lethal dose of HSV-1 or *C. albicans*, the mortality rates of recipient mice were not changed (data not shown).

We have described in the previous reports[15,22] that type-2 cytokines released from burn-associated $CD8^+$ $CD11b^+$ $TCR\gamma/\delta^+$ T type-2 T cells are an effector on the impaired resistance of burned mice to infections of HSV-1 and *C. albicans*. Elimination of IL-4 and IL-10 from thermally injured mice by the administration of a mixture of mAbs for type-2 cytokines caused an improvement in the resistance of these mice to the infections.[15] As the protective effect of BEN or $CD4^+$ T cells from BEN-treated mice is demonstrated only in burned mice, the interactions between burn-associated $CD8^+$ type-2 T cells (or their cytokine products) and $CD4^+$ T cells induced by BEN are suggested when the impaired resistance of thermally injured mice to the infection is restored by BEN. Further experiments are required to explain the mechanism of action on the protective activity of BEN in thermally injured mice infected with HSV-1 or *C. albicans*.

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