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Effects of *Lactobacillus casei*-Containing Ointment on the Healing and Protection against Opportunistic Infection of Thermal Injury Wounds in Mice

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ABSTRACT

I studied the effects of LC ointment made of Solbase and heat-killed *Lactobacillus casei* YIT 0003 on the healing of thermal injury wounds and the elimination of bacteria from infected wounds in mice. LC ointment promoted the healing of the uninfected thermal injury wounds and thermal injury wounds infected with *Pseudomonas aeruginosa* alone or with mixed bacterial suspension of *Staphylococcus aureus, Escherichia coli* and *P. aeruginosa* and resulted in the elimination of infecting bacteria. Histopathologically, the formation of collagen fibers in wounded subcutaneous tissues to which LC ointment was applied was more remarkable than that of tissues treated with Solbase (ointment base). The clearance of bacteria and promotion of healing of the infected thermal injury wounds due to LC ointment were superior to those of Eksalb ointment, Azunol ointment and Mafatate (mafenide acetate) cream, and almost the same degree as that of Geben (silver sulfadiazine) cream. These effects of *L. casei* were nearly the same or somewhat superior compared with that of other lactobacilli, and were enhanced by the addition of gentamicin or ofloxacin.

The topical therapy of thermal injury is usually done for protection of the wound, prevention of infection and promotion of healing. The prevention of infection of burn wounds influences greatly the prognosis of patients. It is thought that the application of topical therapy with ointments containing antibiotics or antibacterial agents is more effective than systemic administration of antibiotics, especially in full thickness extensive burns[2,17,30]. However, in spite of the development of many antiobiotics in recent years, satisfactory effects have not always been achieved, especially against *Pseudomonas aeruginosa*.

We previously reported that *Lactobacillus*, especially *L. casei* has the ability to enhance host resistance to infection due to extracellular[34,35], and intracellular parasites[33,36] in mice, and also clarified that this is due to the accumulation of macrophages at the infected site and acceleration of their function[35,36].

It was considered that the activity of *L. casei* as an immunostimulant could be useful for the promotion of healing and/or prevention of infection of burn wounds. Thus, I studied the effects of *L. casei*-containing ointment (LC ointment) on prevention and healing of wounds infected with opportunistic pathogens, especially with *P. aeruginosa* in experimental burn wounds in mice.

MATERIALS AND METHODS

Animals

Five-week-old female ddY mice weighing 20 ± 2 g were purchased from the Shizuoka Union for Experimental Animals, Shizuoka, Japan. The animals were fed, kept in an air-conditioned

room and given food (MF, Oriental Yeast Co., Tokyo) and water *ad libitum*.

Burned animals

The dorsum of mice anesthetized by intraperitoneal injection of thiopental sodium (63 mg/kg; Tanabe Pharmaceutical Co., Osaka, Japan) were clipped, and a trowel (2 × 3 cm) (Fig. 1) heated in a Bunsen burner was put in its central part for 3 sec. The full thickness burn covering 10% of the body surface was produced by this method, and the calculation of percentage of burned area was done by the method of Freireich et al.[14].

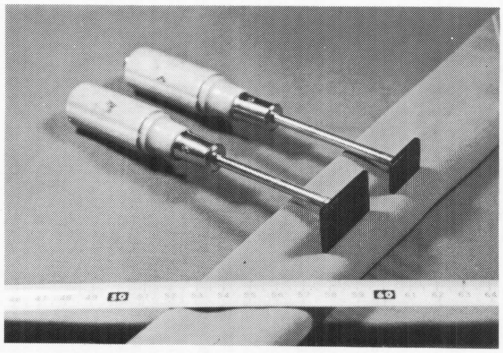

Fig. 1. Photograph of trowels used for preparation of thermal injury wounds in mice.

Bacteria

L. casei YIT 0003 grown in Rogosa's broth[8] at 37°C for 18 hr was washed twice with saline, suspended in saline and heat-killed at 80°C for 30 min. *P. aeruginosa* PAO 3047 was employed as the challenge organism, but each strain of *Staphylococcus aureus* (originated from pus) and *Escherichia coli* (originated from urine) isolated freshly from clinical materials was also employed in some experiments. *P. aeruginosa* and *E. coli* grown in heart infusion broth (Eiken Chemical Co., Tokyo) and *S. aureus* grown in trypto-soy broth (Eiken Chemical Co.) at 37°C for 18 hr were washed twice with saline and suspended in saline.

Ointments for burn wound

LC ointment was prepared by mixing at a ratio of 4:1, Solbase® ointment (Macrogol ointment, Dainippon Pharmaceutical Co., Osaka, Japan; sterilized at 121°C for 15 min) and heat-killed *L. casei* suspended in saline (1×10^{10}/ml). In some experiments, *L. acidophilus* ATCC 19992 (YIT 0075), *L. bulgaricus* ATCC 11842 (YIT 0181), *L. buchneri* ATCC 4005 (YIT 0079), *L. fermentum* ATCC 9338 (YIT 0129), *L. lactis* ATCC 12315 (YIT 0086), *L. plantarum* ATCC 8014 (YIT 0101) or *L. salivarius* ATCC 11739 (YIT 0079) instead of *L. casei* was employed as an immunostimulant. Furthermore, 0.5 g of LC ointment containing 1 or 3 mg of piperacillin (Toyama Chemical Co., Tokyo), cefoperazone (Taito Pfizer Co., Tokyo), gentamicin (Shionogi Pharmaceutical Co., Osaka), ofloxacin (Daiichi Pharmaceutical Co., Tokyo) or AT-2266 (Dainippon Pharmaceutical Co.) were also employed. In order to evaluate the efficacy of LC ointment on burn wounds, the following commercially available ointments were comparatively employed; Eksalb® ointment (Maruho Co., Osaka), Azumol® ointment (Nippon Shinyaku Co., Kyoto, Japan), Silverden® (Geben®) cream (silver sulfadiazine; Tokyo-Tanabe Pharmaceutical Co., Tokyo) and Sulfamylon® (Mafatate®) cream (mafenide acetate; Torii & Co., Tokyo).

Criteria for therapeutic effects of ointments

In experiments with uninfected burn wounds, the eschar was carefully removed with sterilized pincette and scissors 24 hr after thermal injury, and 0.5 g of LC ointment or 0.5 g of Solbase ointment (control) was applied to the wound surface with a sterilized spatula. In the experiments on infected thermal injury, ointment (0.5 g) was applied on the eschar removed wound surface, and 0.1 ml of bacterial suspension (1×10^9/ml) of *P. aeruginosa* PAO 3047 or 0.1 ml of the mixed bacterial suspension of *P. aeruginosa, E. coli* and *S. aureus* (1×10^9/ml each for multiple infections) was inoculated onto the wound surface 24 hr after the application of ointment. In both experiments with or without infection, the wound surface was sketched on tracing paper once a week after initiating the treatment, and its area was measured with a modulator system for semiautomatic quantitative evaluation of images (MOP-AM 03, Kontron Bildanalyse Co., W. Germany). The days required for complete healing of the wound were also determined. In the case of infected burn wounds, the infected wound surface was wiped off with a sterilized cotton swab immersed into sterilized saline (2 ml) every 7 days after infection, and this swab was shaken in 2 ml of saline to free the attached organisms. Each sample was seri-

ally diluted 10-fold with saline, and 0.2 ml of each was plated onto a nalidixic acid-cetrimide (NAC) agar (Eiken Chemical Co.) plate for *P. aeruginosa*, a desoxycholate agar (Eiken Chemical Co.) plate for *E. coli* or a Staphylococcus 110 agar (Eiken Chemical Co.) plate for *S. aureus*, and spread with a Conradi rod. The number of colony-forming units (CFUs) recovered were counted 24 hr after incubation at 37°C. The number of CFUs per cm^2 of burned area was calculated by dividing the number of CFUs by the burned area.

On the other hand, tissues immediately after thermal injury and LC ointment- or Solbase ointment-applied (control) tissues 21 days after thermal injury (3 mice for each group) were excised and fixed with 10% formalin solution. The sections were stained with hematoxylin and eosin or Masson's technique, and observed by microscopy.

RESULTS

Effects of L. casei on healing of thermal injury

As shown in Fig. 2, the wound areas in the group applied with Solbase ointment (Solbase group) and the non-applied group (control group) decreased with time after injury, and no significant differences were noted in both groups through the entire experiment. Although no significant differences were noted in the wound area between the group applied with LC ointment (LC group) and the above two groups at the 7th and 14th day after thermal injury, a significant reduction ($p<0.05$) was noted in LC group at the 21st (Fig. 3) and 28th day. The time required for complete healing of the wound in Solbase, control and LC groups was 29.7 ± 1.1, 31.5 ± 1.2 and 26.0 ± 1.2 days ($p<0.05$), respectively.

Effects of L. casei on clearance of P. aeruginosa from infected burn wounds and healing of wounds

The time courses of the number of CFUs of *P. aeruginosa* and of wound areas in each experimental group (LC, Solbase and control groups) are shown in Figs. 4-A and -B. No significant differences were noted in the number of CFUs and wound areas between control and Solbase groups through the entire experiment. The differences in the number of CFUs and wound areas were hardly noticeable among LC,

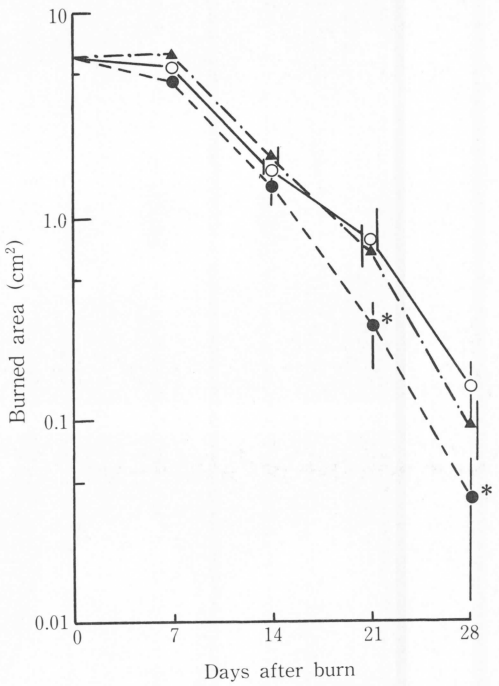

Fig. 2. Changes in the uninfected burned area caused by topical administration of LC ointment.

A full thickness burn (about 10% of total body surface) was induced with an iron trowel (2 × 3 cm) heated in a Bunsen burner flame, to the clipped dorsum of 5-week-old anesthetized female ddY mice and 0.5 g LC ointment (10^9 of heat-killed *L. casei* + Solbase) or 0.5 g Solbase alone was spread over the eschar removed wound 24 hr after burn. Every seven days, the shape of the wound was transcribed onto tracing paper and the area (cm^2) was measured with a modulator system, as described in the text. None (○———○), LC ointment (●– – –●), Solbase alone (▲–·–·–▲). p values were calculated by Student's *t*-test. Bar: mean ± S.E. (n=10), *$p<0.05$ against untreated group.

Solbase and control groups 7 days after infection. They decreased more significantly in the LC group than in the control group 14 days after ($p<0.05$), and than in the Solbase and the control groups 21 days after ($p<0.05$) infection (Fig. 5). The wounds were healed and bacteria in LC group were not recovered 28 days after infection. In contrast, 4.6 ± 3.3 × 10^2 CFUs (1.5 ± 0.7 log CFUs) in the Solbase group and 1.2 ± 0.2 × 10^3 CFUs (2.0 ± 0.8 log CFUs) in the control group were recovered 28 days after infection (Fig. 4-A) and the wounds in the

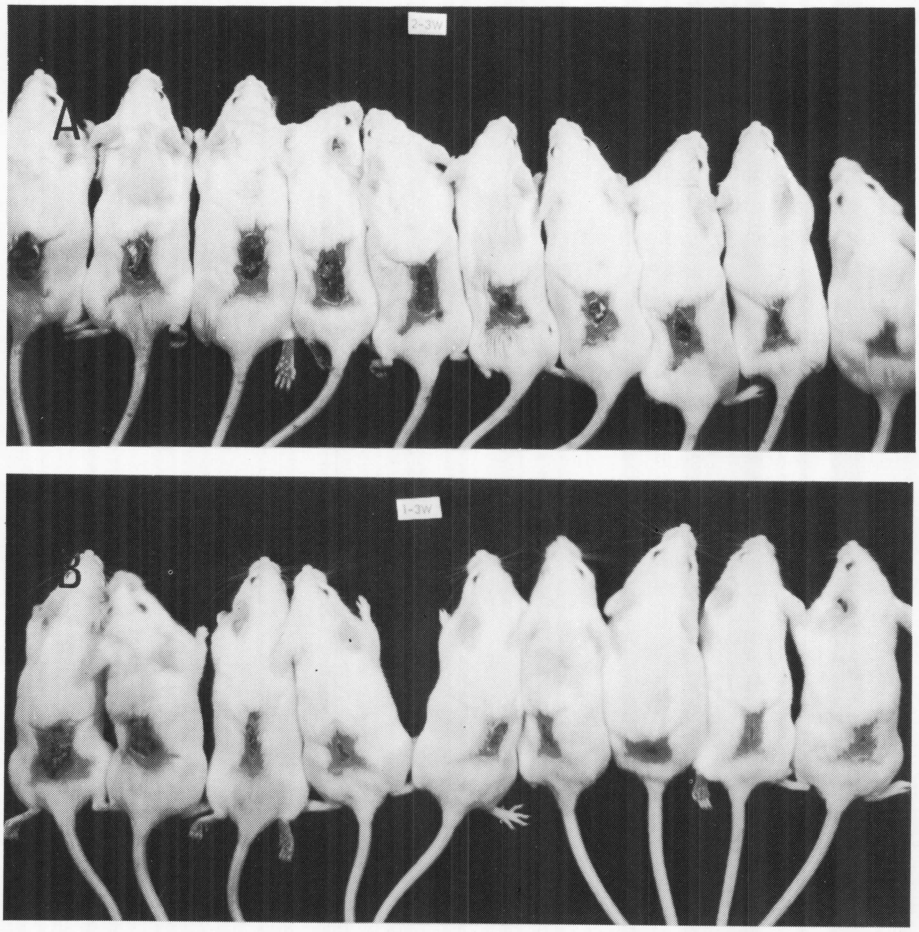

Fig. 3. Photograph of the healing of burn wounds in mice 21 days after burn.
A. Solbase ointment-applied control group. B. LC ointment-applied experimental group.

Solbase and control groups were 0.05 ± 0.03 and 0.04 ± 0.02 cm^2 at that time, respectively (Fig. 4-B). The time required for healing of the infected wound in the Solbase and control groups was 30.3 ± 1.2 and 29.0 ± 1.6 days, respectively, while the time in LC group was 25.1 ± 1.1 days ($p<0.05$).

Comparison between LC ointment and commercially available ointments for burn wounds

The time course of the number of CFUs from wounds infected with *P. aeruginosa* in LC applied group and each group applied with commercially available ointments for burn wounds (Eksalb ointment, Azunol ointment and Geben cream) and in the non-applied control group are shown in Fig. 6-A. Rather many CFUs were noted in Azunol group compared with those in the control group until 14 days after infection, but became somewhat fewer, but not significantly so, 21 days after infection in the former. The recovery of bacteria in the Eksalb group was almost the same as that of the control group until 14 days after infection, while significant decreases were noted at the 21st ($p<0.01$) and 28th day ($p<0.05$) in the former. However, bacterial clearance in the Eksalb group was inferior to that of the Geben group. On the other hand, a significant decrease in the number of CFUs was noted in the LC group compared with the commercially available ointment groups through the entire course of the experiment ($p<0.01$). The time course of the reduction of the wound areas in groups applied with the above 4 kinds of ointments and in the non-applied control group is shown in Fig. 6-B. The degree of reduction of wound areas hardly

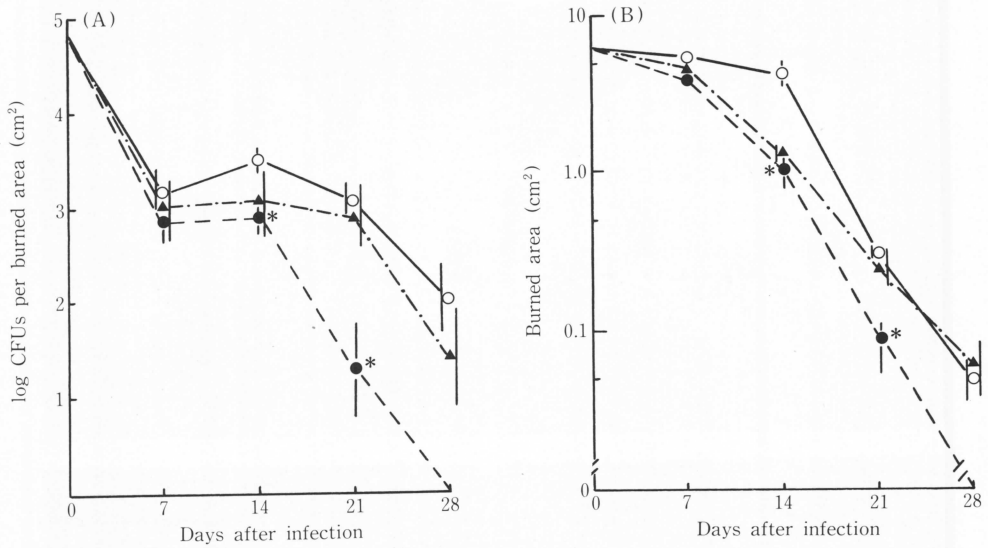

Fig. 4. Effects of LC ointment on burned mice infected with *P. aeruginosa*.

(A) Changes in the number of bacteria in the burn wound. The wound was infected with *P. aeruginosa* PAO 3047 (10^8) 24 hr after topical application of 0.5 g LC ointment or 0.5 g Solbase alone. Every seven days, the infected wound was swabbed and the number of CFUs per cm^2 of the burned area was determined on a NAC agar plate, as described in the text. None (○———○), LC ointment (●– – –●), Solbase alone (▲–·–·–▲). Bar: mean ± S.E. (n=5), *$p<0.05$.

(B) Changes in the burned area. Every seven days, the shape of the wound infected with *P. aeruginosa* was transcribed and the area (cm^2) was measured, as described in the text. None (○———○), LC ointment (●– – –●), Solbase alone (▲–·–·–▲). Bar: mean ± S.E. (n= 6 – 8), *$p<0.05$.

changed in Azunol and Eksalb groups compared with that of control group. On the other hand, significant reduction of wound areas were noted in the Geben group at the 28th day ($p<0.01$), and at the 21st ($p<0.05$) and 28th day ($p<0.01$) in LC group compared with the control group. No significant differences were noted in the time required for complete healing of the wounds between the control group (35.8 ± 1.2 days), Azunol group (36.3 ± 2.1 days) and the Eksalb group (33.9 ± 1.6 days), while significant reductions were noted in the Geben group (33.0 ± 0.5 days; $p<0.05$) and LC group (31.0 ± 1.2 days; $p<0.01$).

The effect exerted on infected burn wounds by Mafatate cream which is being widely employed clinically was compared to that of the LC ointment and Geben cream which were revealed to be the superior ointments for burn wounds (Fig. 7). The decrease in the number of CFUs of *P. aeruginosa* on the infected wound surfaces in the Mafatate group was more remarkable than in the control group, but slightly inferior to the Geben group and remarkably inferior to the LC group (Fig. 7-A). The area of wound reduction progressed almost similarly in the Mafatate and control groups, but a more significant reduction ($p<0.05$) was noted in the LC group and the Geben group at the 28th day after infection than that in control group (Fig. 7-B).

The time required for complete healing of the wounds was hardly different in the Mafatate group (33.6 ± 1.9 days) compared with the control group (34.7 ± 2.1 days), but significant reductions ($p<0.05$) were noted in the Geben group (30.3 ± 1.9 days) and the LC group (29.9 ± 1.4 days).

Clearance of P. aeruginosa from infected burn wounds and effect exerted by ointments containing various lactobacilli on healing of wounds

The time course of the number of CFUs recovered from the *Pseudomonas* infected wound surfaces in animals applied with ointment containing each of 8 species of *Lactobacillus* and non-applied control animals is shown in Fig. 8 (Exps. I and II). The decrease in the number of CFUs of *P. aeruginosa* from the wound area in

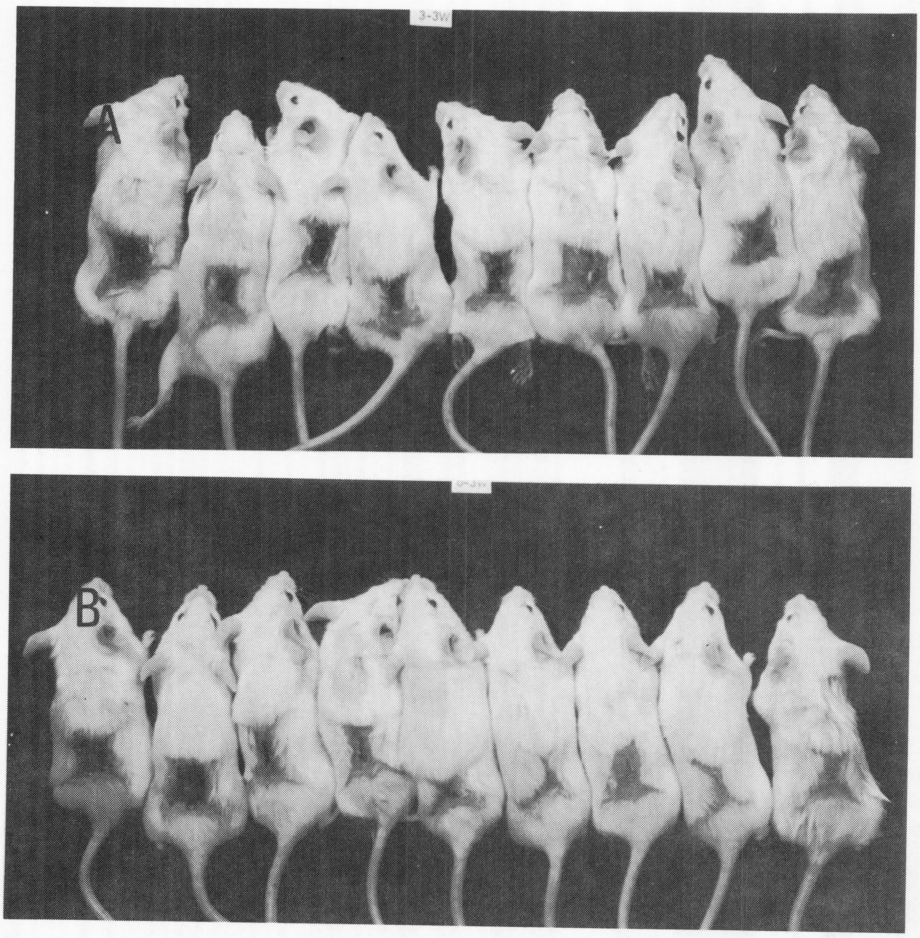

Fig. 5. Photograph of the healing of burn wounds in mice 21 days after infection with *P. aeruginosa* PAO 3047. A. Solbase ointment-applied control group. B. LC ointment-applied experimental group.

the LC group was more remarkable than in the control group, and a significant decrease was noted 14, 21 and 28 days after infection in Exp. I and 21 and 28 days after infection in Exp. II ($p<0.05$). On the other hand, in ointment groups containing *Lactobacillus* other than *L. casei*, the number of CFUs recovered was smaller than in the control group in either, but no significant differences were noted through the entire course of experiment, except for the *L. fermentum* ointment group (Fig. 8-I) and the *L. acidophilus* ointment group (Fig. 8-II) at the 21st day after infection ($p<0.05$). No significant differences were found in the clearance of infected bacteria by species of *Lactobacillus*, but *L. casei* seemed to have somewhat stronger activity than other lactobacilli used in this experiment.

The time course of the wound area in the group applied with ointment containing each of 8 species of *Lactobacillus* and the non-applied control group is shown in Fig. 9 (Exps. I and II). A significant reduction of wound area was noted at the 21st and 28th day in the *L. salivarius* ointment group and at the 21st day in the *L. buchneri* ointment group compared with the control group ($p<0.05$), and a more significant reduction was noted in the LC ointment group than in the other ointment groups ($p<0.01$). On the other hand, no large differences were noted in the *L. lactis* and *L. fermentum* ointment groups compared with the control group through the entire course of the experiment (Fig. 9-I). The degree of the reduction of the wound area was slightly favorable without statistical significance in the *L. bulgaricus*, *L. acidophilus* and *L. plantarum* ointment groups

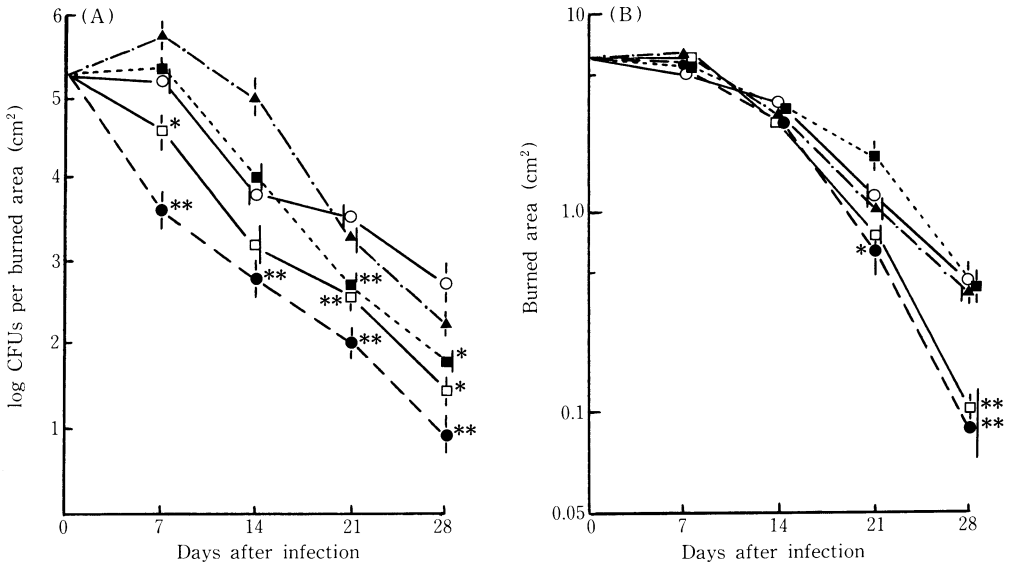

Fig. 6. Comparison of bacteria-eliminating and burn wound-healing effects of LC ointment and commercial ointments on the burn wound infected with *P. aeruginosa*.

(A) Changes in the number of CFUs in the burn wound. The wound was infected with *P. aeruginosa* (10^8) 24 hr after topical application of 0.5 g LC ointment or 0.5 g various commercial ointments. Every seven days, the number of CFUs was assayed. None (○———○), LC ointment (●— —●), Azunol ointment (▲—·—·—▲), Eksalb ointment (■-------■), Geben cream (□———□). Bar: mean ± S.E. (n=5), *p<0.05, **p<0.01.

(B) Changes in the burned area. The area of the burn pretreated with or without ointment and infected was measured every seven days. None (○———○), LC ointment (●— —●), Azunol ointment (▲—·—·—▲), Eksalb ointment (■-------■), Geben cream (□———□). Bar: mean ± S.E. (n=8), *p<0.05, **p<0.01.

compared with that in the control group. On the contrary, a significant reduction ($p<0.05$) was noted at the 21st and 28th day in the LC ointment group compared with that in the control group as well as in the Experiment I (Fig. 9-II).

As shown in Table 1, a significant reduction of time required for complete healing of the wounds was noted in the LC group compared with the control group in Experiments I and II ($p<0.05$). However, significant promoting effects on the healing of wounds was not noted, except for *L. salivarius* (Exp. I), among the *Lactobacillus* (7 species) other than *L. casei*.

Clearance of multiple bacteria from infected burn wounds and the effect on healing of wounds due to LC ointment

Table 1. Time required for complete healing of the burn wound infected with *P. aeruginosa* by topical application of various *Lactobacillus* ointments[a]

Exp. I		Exp. II	
Ointment	mean ± S.E. (n=5) (days)	Ointment	mean ± S.E. (n=10) (days)
None	35.1 ± 1.7	None	28.6 ± 1.5
LC	30.8 ± 2.1*	LC	22.9 ± 1.5*
L. salivarius	30.9 ± 1.7*	*L. plantarum*	24.5 ± 1.6
L. buchneri	31.9 ± 1.2	*L. acidophilus*	25.0 ± 2.2
L. lactis	34.0 ± 2.6	*L. bulgaricus*	25.4 ± 1.3
L. fermentum	32.7 ± 2.3		

[a]The wound was infected with *P. aeruginosa* (10^8) 24 hr after topical application of 0.5 g of various *Lactobacillus* ointments (10^9 of heat-killed bacteria + Solbase). Time required for complete healing of the wound was recorded. *p<0.05.

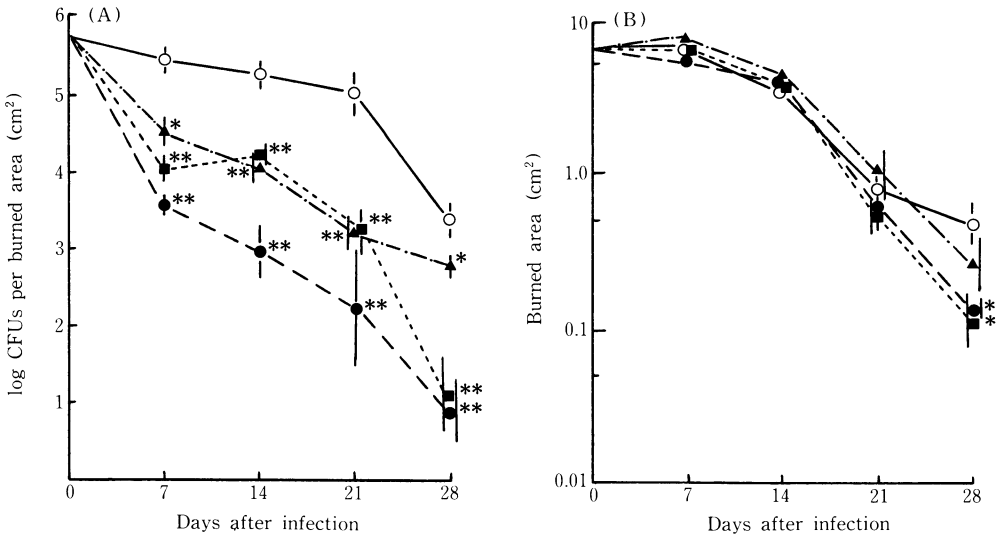

Fig. 7. Comparison of bacteria-eliminating and burn wound-healing effects of LC ointment, Mafatate cream and Geben cream on the burn wound infected with *P. aeruginosa*.

(A) Changes in the number of CFUs in the burn wound. The wound was infected with *P. aeruginosa* (10^8) 24 hr after topical application of 0.5 g LC ointment, 0.5 g Mafatate cream or 0.5 g Geben cream. Every seven days, the number of CFUs was assayed. None (○———○), LC ointment (●— — —●), Mafatate cream (▲- - —- -▲), Geben cream (■- - - - -■). Bar: mean ± S.E. (n=5), *p<0.05, **p<0.01.

(B) Changes in the burned area. The area of the burn wound pretreated with or without ointment and infected was measured every seven days. None (○———○), LC ointment (●— — —●), Mafatate cream (▲- - —- -▲), Geben cream (■- - - - -■). Bar: mean ± S.E. (n=7), *p<0.05.

The time course of the number of CFUs of each organism recovered from the burn injury surface infected with a mixed suspension of *P. aeruginosa*, *E. coli* and *S. aureus* in the LC applied ointment group and non-applied control group is shown in Fig. 10. The number of CFUs of each organism recovered from the infected wound area applied with LC ointment decreased with the time after infection, but the degree of decrease was more remarkable than in the control group; significant decrease was noted in *P. aeruginosa* and *E. coli* at the 14th, 21st and 28th day, and in *S. aureus* at the 7th, 14th, 21st and 28th day after infection ($p<0.05$ or $p<0.01$). The time course of the wound area infected with the above mixed bacterial suspension in the LC and control groups is shown in Fig. 11. No differences were found between both groups until 14 days after infection, but a significant reduction was noted in the LC group compared with the control group at the 21st and 28th day after infection ($p<0.05$).

Clearance of P. aeruginosa from infected burn wounds and effect on healing of wounds due to LC ointment containing anti-P. aeruginosa chemotherapeutics

The time course of the number of CFUs of *P. aeruginosa* recovered from infected burn wounds applied with LC ointment with or without piperacillin, cefoperazone, gentamicin, ofloxacin or AT-2266 is shown in Table 2. The clearance of bacteria from the wound area was enhanced by the addition of 1 mg of gentamicin or ofloxacin to the LC ointment, and a significant decrease in the number of CFUs was noted at the 21st day after infection ($p<0.05$), and bacteria were not recovered at the 28th day. On the other hand, the clearance of bacteria was not enhanced by the addition of 1 mg piperacillin, cefoperazone or AT-2266, but was enhanced by the addition of 3 mg. However, the healing of burn wounds could not be promoted by adding each chemotherapeutic (1 or 3 mg) into the LC ointment.

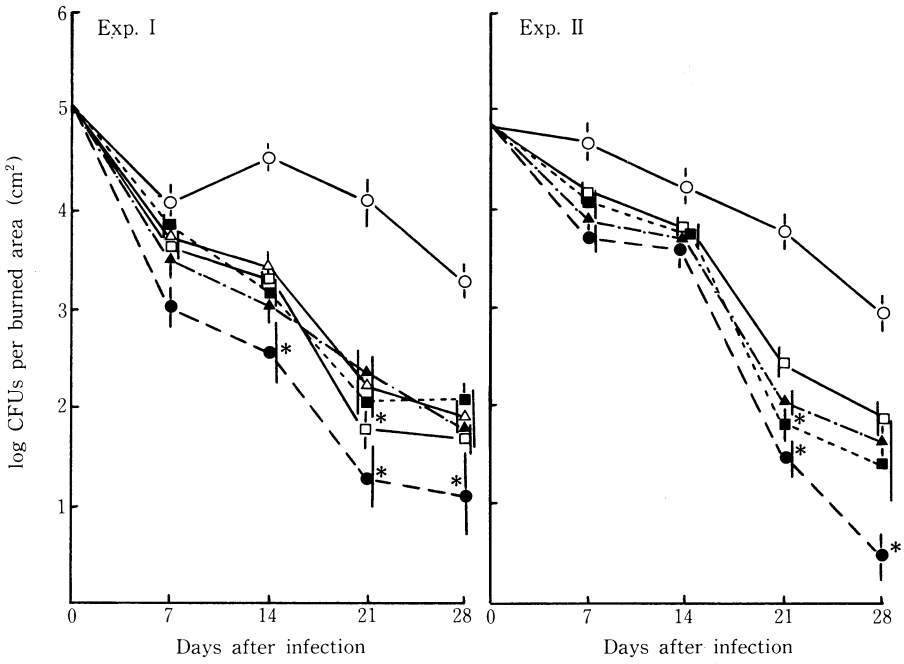

Fig. 8. Changes in the number of *P. aeruginosa* in the burn wound by topical application of various *Lactobacillus* ointments.

The wound was infected with *P. aeruginosa* (10^8) 24 hr after topical application of 0.5 g LC ointment or 0.5 g Solbase containing 10^9 of heat-killed *Lactobacillus* tested (7 species). Every seven days, the number of CFUs was assayed.

Exp. I. None (○———○), LC ointment (●— — —●), *L. salivarius* ointment (▲-·—·-▲), *L. buchneri* ointment (△———△), *L. lactis* ointment (■------■). *L. fermentum* ointment (□———□). Bar: mean ± S.E. (n=5), *$p<0.05$.

Exp. II. None (○———○), LC ointment (●— — —●), *L. plantarum* ointment (▲— ·—·-▲), *L. acidophilus* ointment (■------■), *L. bulgaricus* ointment (□———□). Bar: mean ± S.E. (n=5), *$p<0.05$.

Table 2. Changes in the number of *P. aeruginosa* in the burn wound by topical application of LC ointment containing various chemotherapeutic agents[a]

Additions		log CFUs per burned area (cm^2)			
		7	14	21	28 (days)
None		5.34 ± 0.17	4.70 ± 0.19	3.48 ± 0.28	3.38 ± 0.29
LC ointment		2.93 ± 0.35††	2.58 ± 0.25††	2.61 ± 0.10†	1.03 ± 0.63††
+ Piperacillin	1 mg	3.54 ± 0.21	2.57 ± 0.26	2.13 ± 0.56	1.64 ± 0.69
	3 mg	2.59 ± 0.29	2.10 ± 0.17	1.73 ± 0.44	0
+ Cefoperazone	1 mg	3.18 ± 0.28	2.62 ± 0.25	1.96 ± 0.51	1.15 ± 0.71
	3 mg	2.49 ± 0.36	1.93 ± 0.22	1.32 ± 0.55	0
+ Gentamicin	1 mg	2.25 ± 0.35	2.00 ± 0.12	0.91 ± 0.56*	0
	3 mg	1.03 ± 0.47**	0.21 ± 0.17**	0	0
+ Ofloxacin	1 mg	2.26 ± 0.27	2.02 ± 0.16	0.99 ± 0.53*	0
	3 mg	0.70 ± 0.34**	0.21 ± 0.19**	0	0
+ AT-2266	1 mg	3.24 ± 0.30	2.40 ± 0.25	1.92 ± 0.49	1.45 ± 0.60
	3 mg	2.69 ± 0.32	1.91 ± 0.20	1.55 ± 0.49	0.53 ± 0.53

[a]The wound was infected with *P. aeruginosa* PAO 3047 (10^8) 24 hr after application of 0.5 g LC ointment or 0.5 g LC ointment containing 1 or 3 mg of the chemotherapeutic agent. Every seven days, the number of CFUs was assayed.

†$p<0.05$, ††$p<0.01$ against untreated group; *$p<0.05$, **$p<0.01$ against LC ointment-treated group.

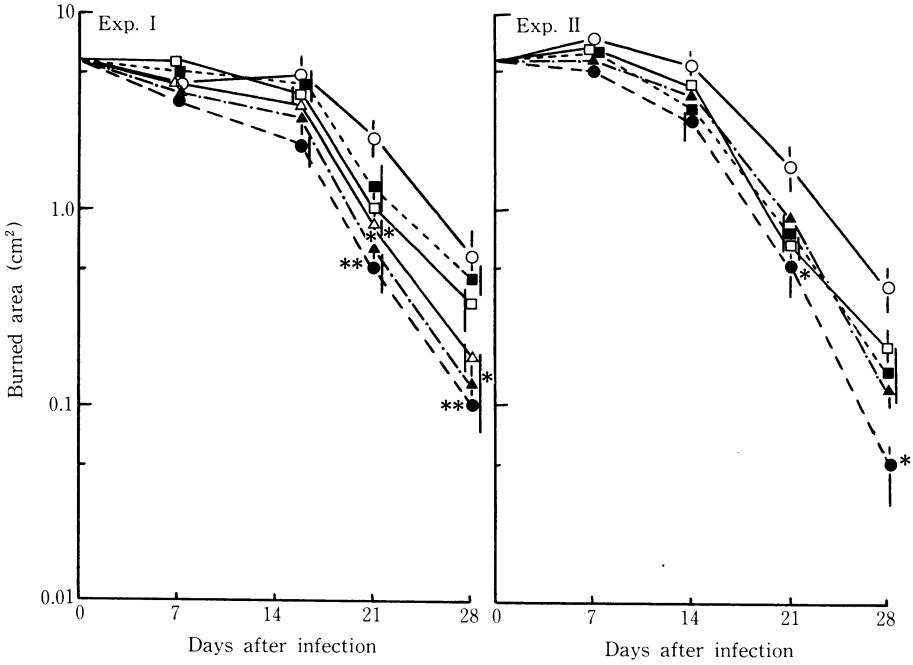

Fig. 9. Changes in the burned area by topical application of various *Lactobacillus* ointments.
The wound was pretreated with or without ointment and infected as described in the legend for Fig. 8. The burned area was measured every seven days.
Exp. I. None (○———○), LC ointment (●— — —●), *L. salivarius* ointment (▲-·—·-▲), *L. buchneri* ointment (△———△), *L. lactis* ointment (■------■), *L. fermentum* ointment (□———□). Bar: mean ± S.E. (n=5), *$p<0.05$, **$p<0.01$.
Exp. II. None (○———○), LC ointment (●— — —●), *L. plantarum* ointment (▲-·—·-▲), *L. acidophilus* ointment (■------■), *L. bulgaricus* ointment (□———□). Bar: mean ± S.E. (n=10), *$p<0.05$.

Histopathology of thermal injury wounds

Fig. 12 shows the photomicrograph of mouse skin immediately after the burn. It was observed that complete coagulation necrosis and necrobiosis of remaining root of hair in epidermis and dermis, and edematous swelling of subcutaneous tissues occurs (hematoxylin and eosin staining). In Masson's staining of wounded tissues 21 days after thermal injury (Fig. 13), fibroblasts accompanied by an infiltration of round cells was observed under the crust, and collagen fibers (stained to blue) were few in the Solbase ointment (control) group (A). On the contrary, in the LC ointment group, at the same period, the formation of epidermis was noted and infiltration of round cells was extremely sparse. The formation of fibrous tissues and of a large amount of collagen fibers was observed (B).

DISCUSSION

The therapy of thermal injury largely differs depending on severity of wounds; mild cases are treated with topical application of ointment alone and extremely severe cases necessitate systemic therapy. Even if a wound by thermal injury is of a small area, its resistance against infection decreases more than healthy skin or mucous membrane. The surface of the erosion in the second degree burn (dermal burn) or eschar in the third degree burn (full thickness burn) provides a favorable growth medium for bacteria and becomes a focus of burn wound sepsis. Systemic effects are hardly noted in mild cases of thermal injury even though infection occurs on the wound, while impairment of host immune defense mechanisms develops in cases of extensive heavy thermal injury[1,5–7,9,18,21–23,25,26,31] and infection easily develops in the wound. Once infections, especially infection with *P. aeruginosa* occurs, they occasionally progress to sepsis or pneumonia[17,38,40]. Therefore, needless to say, the prevention of infection of wounds markedly in-

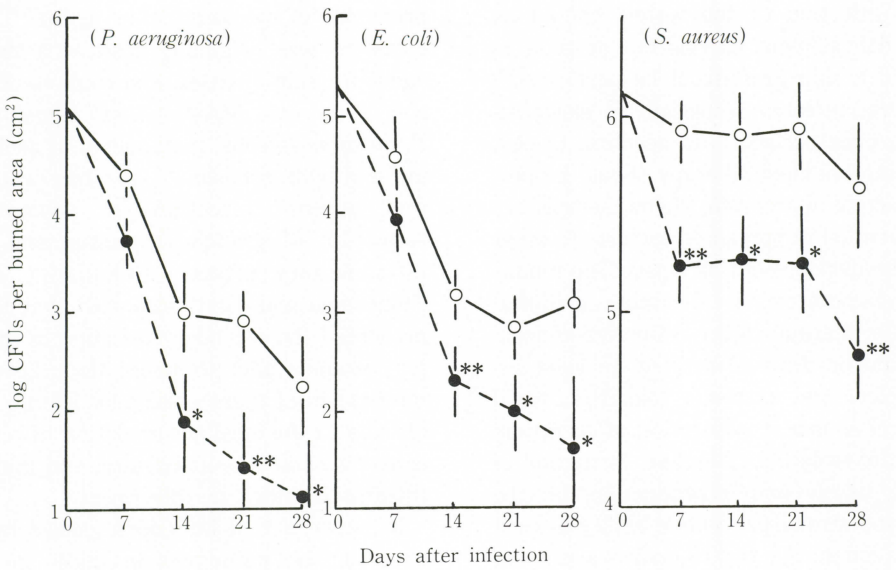

Fig. 10. Changes in the number of bacteria in the mixed-infected burn wound by topical application of LC ointment. The wound was infected with the mixture of *P. aeruginosa*, *E. coli* and *S. aureus* (10^8 of each organism) 24 hr after topical application of 0.5 g LC ointment. Every seven days, the number of *P. aeruginosa*, *E. coli* and *S. aureus* was determined on a NAC agar plate, a desoxycholate agar plate and a Staphylococcus 110 agar plate, respectively. None (○——○), LC ointment (●– – –●). Bar: mean ± S.E. (n=5), *p<0.05, **p<0.01.

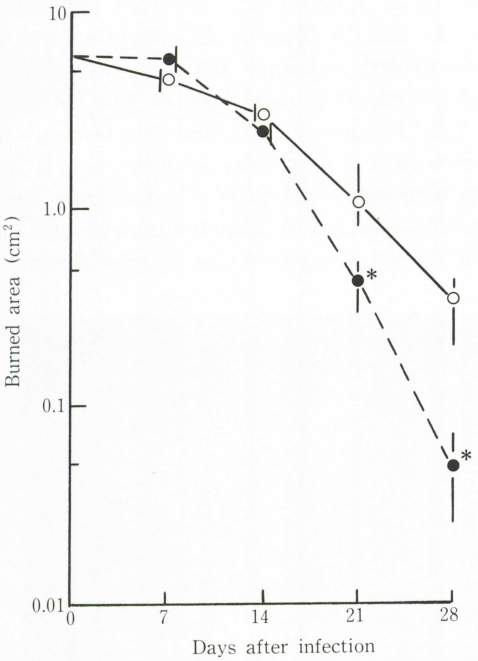

Fig. 11. Changes in the burned area in the mixed-infected burn wound by topical application of LC ointment.

The wound was pretreated with or without ointment and infected as described in the legend for Fig. 7. The burned area was measured every seven days. None (○——○), LC ointment (●– – –●). Bar: mean ± S.E. (n=10), *p<0.05.

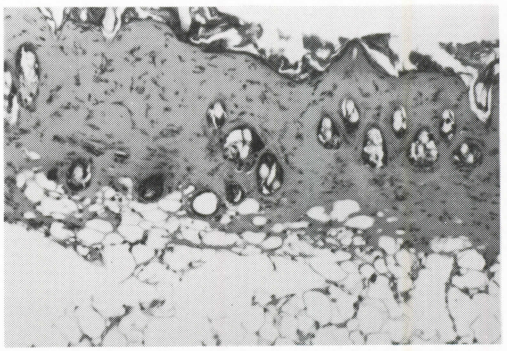

Fig. 12. Photomicrograph of mouse wound tissue immediately after the burn showing complete coagulation necrosis and necrobiosis of remaining root of hair in the epidermis and dermis, and edematous swelling of subcutaneous tissues (hematoxylin and eosin staining; original magnification: × 100).

fluences the prognosis of patients. With a local burn wound having been established as the source of a burn wound sepsis and the avascular nature of this same wound obviating effective systemic therapy, it becomes obvious that the only effective route of the therapy is the topical one. As a rule of topical therapy, the wound surface should be aseptically protected and epithelization should be aided in second degree

burns, and infection of the wound should be prevented, débridement carried out as soon as possible, and a skin graft must be performed[3]. When bacterial infection, especially *P. aeruginosa* infection once occurs, it is needless to say, systemic intensive chemotherapy should be performed, selecting appropriate chemotherapeutics for the prevention of spread of bacteria to other organs or the development of sepsis. The following various factors may be desirable in topical ointments for thermal injury: effective concentration should be diffused actively to local tissue; no topical and systemic toxicities; rapid excretion and abundant adsorption of secretion; acceleration of isolation of eschar, formation of granulation tissue and reproduction of the epidermis; and strong preventive ability against bacterial infection. As the causative agents of infection in thermal injury wounds, *S. aureus* and *P. aeruginosa* are typical representatives of gram-positive bacteria and gram-negative bacteria, respectively. *P. aeruginosa* infection occurs rarely in the early stage of the injury and occurs as a mixed infection or superinfection during the therapy[39]. As the topical therapeutic agents, especially for prevention of *P. aeruginosa* infection, gentamicin cream[24], silver nitrate solution[28], mafenide acetate cream[27] and silver sulfadiazine cream[11,13] are excellent. Mafenide acetate cream produces a local burning sensation of varying severity at the time of application, but sepsis due to *P. aeruginosa* became preventable by using this agent[20,29]. Eksalb ointment was originally used as a therapeutic agent for skin diseases, and consists of killed *E. coli*, *S. aureus*, *Streptococcus hemolyticus* and *P. aeruginosa* cells, metabolites of each bacterium and hydrocortisone[37], and has protective effect against infection by promoting the formation of granulation tissue and an anti-inflammatory effect[4]. Fukuda et al.[15] and Fukushima and Yagi[16] reported that this agent prevented the secondary infection of thermal injury wounds and promoted their healing. The mechanism of these effects by Eksalb is probably due to the local accumulation of phagocytes caused by the various bacteria and their culture filtrates included in this agent.

In experimental infections caused by various opportunistic pathogens in mice, we clarified previously that *L. casei* YIT 0003 enhanced host resistance and this is largely attributable to the accumulation of phagocytes at the infection site[35,36]. Recently, we further clarified that this activity exists in the cell wall fraction (unpublished data). Kato et al.[19] reported that heat-killed *L. casei* YIT 9018 (LC 9018) showed a strong growth-inhibitory action in transplantation experiments in mice with sarcoma 180 cells and L1210 leukemia cells, and this was due to the activation of macrophages by this agent. The above-mentioned effects of *L. casei* were similar to those caused by Eksalb ointment, and the possibility was suggested that *L. casei*-combined

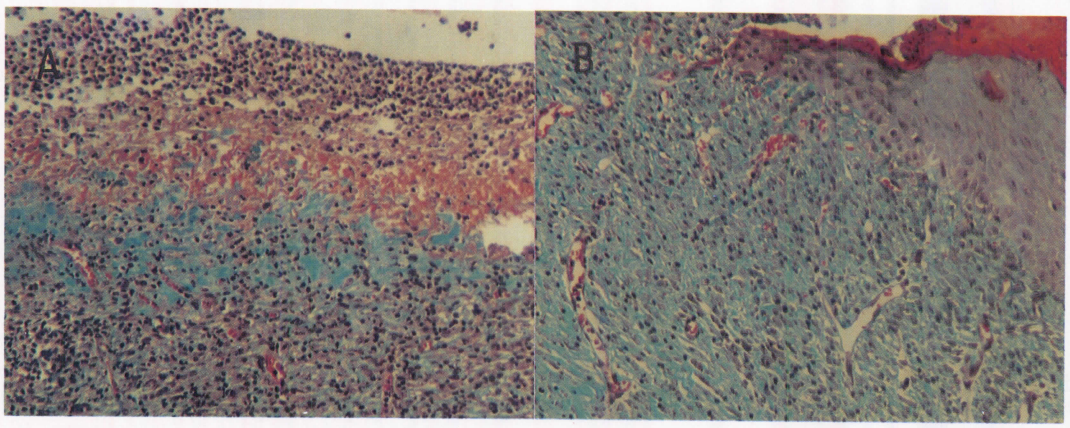

Fig. 13. Photomicrograph of mouse wound tissue 21 days after the burn. The infiltration of fibroblasts accompanying by round cells, and few collagen fibers (stained blue) were observed under the crust in the control (A); In LC ointment group (B) in the same period, the formation of epidermis, infiltration of a few round cells, the formation of fibrous tissues and of a large amount of collagen fibers were observed (Masson's staining; original magnification: × 100).

ointment can be employed as a topical therapeutic agent for thermal injury.

We clarified the following important points for employing an ointment containing *L. casei* YIT 0003 (LC ointment) for treatment of thermal injury in mice. Firstly, the healing of wounds is accelerated by the promotion of formation of granulation tissue in thermal injury wounds. Secondly, the bacterial clearance from the wound surface infected with *P. aeruginosa* or with a mixed suspension of *S. aureus*, *E. coli* and *P. aeruginosa* and the healing of the wounds were stimulated. Thirdly, the above effects were noted with various lactobacilli but the strongest was *L. casei*. The clearance of *P. aeruginosa* from the wound surface was enhanced by the addition of gentamicin or ofloxacin to the LC ointment. It is believed that the beneficial effects of application of LC ointment on uninfected and infected thermal injury wounds are due to the promotion of the accumulation of phagocytes at the wound site, and the accumulated phagocytes phagocytize and clear the bacteria and/or necrotic tissues, and thus, hasten the formation of granulation tissue which causes the healing of wounds. This was supported by the histopathological findings (Fig. 13). For the elimination of bacteria and promotion of healing, LC ointment is superior to mafenide acetate (Mafatate) cream which is especially effective for *Pseudomonas* infections as a topical therapeutic agent for deep dermal burn or full thickness burn, and almost equal to silver sulfadiazine (Geben) cream. In the present experiments with *Pseudomonas* infected thermal injured mice, bacteria were gradually cleared and decreased with time course after infection even in the untreated control mice. Therefore, a study employing a burn wound sepsis model[10,12,32] would be desirable in order to further correctly evaluate the effects of LC ointment.

L. casei has been utilized for food, etc., since long ago and is generally regarded as saprophytic. In fact, it seems that the acute and chronic toxicities of *L. casei* YIT 9018 are extremely low[19].

From the above results, LC ointment seems potentially to be one of the therapeutic ointments for thermal injury in future, because of its excellence in stimulating the clearance of infected bacteria and promoting healing of wounds.

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REFERENCES

1. **Alexander, J.W. and Wixson, D.** 1970. Neutrophil dysfunction and sepsis in burn injury. Surg. Gynecol. Obstet. **130**: 431—438.
2. **Anzai, T.** 1983. Local treatment of burns. Asian Med. J. **26**: 189—198.
3. **Anzai, T. and Tomizawa, T.** 1973. Local treatment of third-degree of burns. Surg. Ther. **29**: 538—545.
4. **Aoki, T. and Tamai, T.** 1974. Role of leucocytes in inflammation — chemotactic, phagocytic and bactericidal effects of killed bacterial suspensions of Posterisan and Eksalb for leucocytes —. Basic Pharmacol. Therapeutics **2**: 1463—1472.
5. **Arturson, G., Högman, C.F., Johansson, S.G.O. and Killander, J.** 1969. Changes in immunoglobulin levels in severely burned patients. Lancet **1**: 546—548.
6. **Balch, H.H., Watters, M. and Kelley, D.** 1963. Resistance to infection in burned patients. Ann. Surg. **157**: 1—19.
7. **Bjornson, A.B. and Alexander, J.W.** 1974. Alterations of serum opsonins in patients with severe thermal injury. J. Lab. Clin. Med. **83**: 372—382.
8. **Efthymiou, C. and Hansen, P.A.** 1962. An antigenic analysis of *Lactobacillus acidophilus*. J. Infect. Dis. **110**: 258—267.
9. **Fjellström, K.E. and Arturson, G.** 1963. Changes in the human complement system following burn trauma. Acta Path. Microbiol. Scand. **59**: 257—270.
10. **Fox, C.L., Jr.** 1952. Electrolyte solution approximating plasma concentrations. J.A.M.A. **148**: 827-833.
11. **Fox, C.L.,Jr.** 1968. Silver sulfadiazine — a new topical therapy for *Pseudomonas* in burns. Arch. Surg. **96**: 184—188.
12. **Fox, C.L., Jr. and Stanford, J.W.** 1965. Toxic and anti-sodium action of thromboplastic extracts of skin and muscle in burn and tourniquet trauma in mice. Life Sci. **4**: 215—224.
13. **Fox, C.L., Jr., Rappole, B.W. and Stanford, J.W.** 1969. Control of *Pseudomonas* infection in burns by silver sulfadiazine. Surg. Gynecol. Ob-

stet. **128**: 1021—1026.

14. **Freireich, E.J., Gehan, E.A., Rall, D.P., Schmidt, L.H. and Skipper, H.E.** 1966. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey and man. Cancer Chemother. Reports **50**: 219—244.
15. **Fukuda, K., Nagasaki, T. and Yamaguchi, M.** 1968. Topical treatment of burn wounds with Eksalb ointment. Med. Treatment **1**: 990—993.
16. **Fukushima, H. and Yagi, Y.** 1973. Topical treatment of various burn wounds with Eksalb ointment. J. New Remed. Clin. **22**: 499—505.
17. **Hayashi, S.** 1973. Problems on severe thermal injury. Surg. Ther. **29**: 496—500.
18. **Ishibiki, H., Kawahara, H., Murayama, N., Yagita, A., Takami, H., Ikeuchi, T., Ushijima, K., Aikawa, N., Abe, N. and Sumimoto, Y.** 1977. Immunodeficiency in burned patients. Jpn. J. Burn Injury **2**: 244—251.
19. **Kato, I., Kobayashi, S., Yokokura, T. and Mutai, M.** 1981. Antitumor activity of *Lactobacillus casei* in mice. Gann **72**: 517—523.
20. **Lindberg, R.B., Brame, R.E., Moncrief, J.A. and Mason, A.D.** 1964. Prevention of invasive *Pseudomonas aeruginosa* infections in seeded rats by use of topical sulfamylon cream. Fed. Proc. **23**: 388.
21. **Loose, L.D., Megirian, R. and Turinsky, J.** 1984. Biochemical and functional alterations in macrophages after thermal injury. Infect. Immun. **44**: 554—558.
22. **Loose, L.D. and Turinsky, J.** 1979. Macrophage dysfunction after burn injury. Infect. Immun. **26**: 157—162.
23. **Lowbury, E.J.L., Ricketts, C.R., Hall, M., Hurst, L. and Lilly, H.A.** 1959. Properdin and the defence of burns against infection. J. Hyg. **55**: 266—275.
24. **MacMillan, B.C.** 1973. Critique of the current status of burn sepsis control. "Symposium of the treatment of burns", Vol. V, 140, the C.V. Mosby Co., St. Louis, U.S.A.
25. **MucEuen, D.D., Blair, P., Delbene, V.E. and Eurenius, K.** 1976. Correlation between *Pseudomonas* burn wound infection and granulocyte antibacterial activity. Infect. Immun. **13**: 1360—1362.
26. **MucEuen, D.D., Gerber, G.C., Blair, P. and Eurenius, K.** 1976. Granulocyte function and *Pseudomonas* burn wound infection. Infect. Immun. **14**: 399—402.
27. **Moncrief, J.A., Lindberg, R.B., Switzer, W.E. and Pruitt, B.A., Jr.** 1966. The use of a topical sulfonamide in the control of burn wound sepsis. J. Trauma **6**: 407—419.
28. **Moyer, C.A., Brentano, L., Gravens, D.L., Margraf, H.W. and Monafo, W.W., Jr.** 1965. Treatment of large human burns with 0.5% silver nitrate solution. Arch. Surg. **90**: 812—867.
29. **Muramatsu, M.** 1973. Chemotherapy of burns with sulfamylon. Surg. Ther. **29**: 546—551.
30. **Ohura, T.** 1977. Local treatment of burns. Orthopae. Trauma. Surg. **20**: 819—827.
31. **Rittenbury, M.S. and Hanback, L.D.** 1967. Phagocytic depression in thermal injuries. J. Trauma **7**: 523—540.
32. **Rosenthal, S.M.** 1967. Local and systemic therapy of *Pseudomonas* septicemia in burned mice. Ann. Surg. **165**: 97—103.
33. **Saito, H., Tomioka, H. and Sato, K.** 1981. Protective effects of various lactobacilli on *Listeria* infection in mice. Med. Biol. **102**: 273—277.
34. **Saito, H., Watanabe, T., Tomioka, H., Horikawa, Y. and Tado, O.** 1980. Increased host resistance to *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* infections by *Lactobacillus casei*. Med. Biol. **100**: 285—288.
35. **Saito, H., Watanabe, T. and Horikawa, Y.** 1986. Effects of *Lactobacillus casei* on *Pseudomonas aeruginosa* infection in normal and dexamethasone-treated mice. Microbiol. Immunol. (in press).
36. **Sato, K.** 1984. Enhancement of host resistance against *Listeria* infection by *Lactobacillus casei*: role of macrophages. Infect. Immun. **44**: 445—451.
37. **Takahashi, K., Fujimoto, S., Sasaki, N., Tamai, T., Yagi, M., Gotho, M., Nagaoka, M. and Yoshida, T.** 1977. Pharmacological actions of Eksalb. Basic Pharmacol. Therapeutics **5**: 397—406.
38. **Teplitz, C., Davis, D., Mason, A.D.,Jr. and Moncrief, J.A.** 1964. *Pseudomonas* burn wound sepsis. I. Pathogenesis of experimental burn wound sepsis. J. Surg. Res. **4**: 200—216.
39. **Tomizawa, T.** 1981. *Pseudomonas aeruginosa* infection in burns. Japan Med. Association **36**: 1731—1737.
40. **Walker, H.L., Mason, A.D.,Jr. and Raulston, G.L.** 1964. Surface infection with *Pseudomonas aeruginosa*. Ann. Surg. **160**: 297—305.