Clin. exp. Immunol. (1979) 37, 367–375.

Adjuvanticity of lactobacilli

I. DIFFERENTIAL EFFECTS OF VIABLE AND KILLED BACTERIA

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(*Accepted for publication* 13 *December* 1978)

SUMMARY

The adjuvanticity of *Lactobacillus brevis* and *Lactobacillus plantarum* was the subject of this study. The latter was the better adjuvant in both delayed hypersensitivity and antibody formation to sheep red blood cells. Viable *L. plantarum* stimulated exclusively the delayed hypersensitivity, where heat-killed bacteria had an adjuvant effect on antibody formation. For optimal adjuvant effects lactobacilli had to be injected in a dose of 10^8 into the same site as the antigen. Viable lactobacilli and to a lesser degree heat-killed bacteria induce hepato-splenomegaly, suggesting mediation of the adjuvant activity by the reticuloendothelial system. Granuloma formation with mainly mononuclear cell infiltrates could be observed after subcutaneous administration of viable lactobacilli whereas heat-killed lactobacilli induced granulomata containing about equal numbers of granulocytes and mononuclear cells. The possible clinical application of *L. plantarum* in the immunotherapy of tumours is suggested.

INTRODUCTION

The possible immuno-enhancing effect of two species of lactobacilli was the subject of this study. They were isolated from Iscador®, a preparation from mistletoe which is registered in Switzerland and has been used in cancer therapy since 1921. Iscador® is made by natural fermentation of fresh plant juice, which comes to contain a high content of lactobacilli. Tumour growth inhibition by Iscador® *in vivo* and *in vitro* has been reported in mouse systems (Selawry, Schwartz & Haar, 1959; Selawry & Schwartz, 1961; Evans & Preece, 1973). Moreover Iscador® induces splenomegaly and stimulation of the reticulo-endothelial system (Zschiesche, 1966). These effects of Iscador® are probably in part due to immuno-potentiation (Bloksma *et al.*, 1979). As many bacterial species can enhance the immune response to an unrelated antigen or stimulate a non-specific resistance to intracellular parasites and tumours (reviewed by Campbell, 1976; Borek, 1977), we investigated the adjuvant effect of two species of lactobacilli isolated from Iscador®.

In general, lactobacilli are considered non-pathogenic, except perhaps *L. catenaforme* (Smith, 1975). Multiplication *in vivo* is thought to be unlikely but survival for 1 week in rabbits has been described. We tested both viable and heat-killed lactobacilli in mineral oil and aqueous suspensions. As granuloma formation is a property of many adjuvants (Boros, 1978; Borek, 1977) the granuloma-inducing capacity of viable and heat-killed lactobacilli has been examined.

MATERIALS AND METHODS

Animals. Swiss mice were obtained from the Central Institute for the Breeding of Laboratory Animals (CPB, Zeist, The Netherlands). Female mice were used at an age of about 14 weeks.

Antigen. Sheep red blood cells (SRBC), stored in Alsever's old solution, were obtained from the National Institute of Public Health (R.I.V., Bilthoven, The Netherlands). Before use the SRBC were washed three times with sterile saline.

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0099-9104/79/0800-0367$02.00

Lactobacilli. Two species of lactobacilli were isolated from Iscador® (Hiscia; Arlesheim, Switzerland) and identified as *L. plantarum* and *L. brevis* by Dr J.A.A. Hoogkamp-Korstanje (Laboratory of Microbiology, Utrecht). The bacteria were grown under anaerobic conditions in De Man-Rogosa-Sharpe (MRS) broth (Oxoid Ltd., London) at 37°C for 16 hr. The bacteria were washed three times and resuspended in sterile saline and counted photometrically in a Klett Summerson nephelometer. Fresh bacteria were diluted to the desired concentration and injected as such or after killing by incubation for 1 hr at 56°C.

In some experiments lactobacilli which were freshly harvested, washed and resuspended in MRS medium were used. Thest were kept deep-frozen at −70°C. The percentage of viable bacteria in such deep-frozen preparations remained constant ae 45% for several months as counted by the method of Miles & Misra (1938).

Mineral oil adjuvants. Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were obtained from Difco Laboratories (Detroit, Michigan). FIA consists of 85% Bayol F and 15% Arlacel A (v/v), FCA contains in addition 0·5 mg of killed *Mycobacterium butyricum* per ml FIA. Antigen solution (0·1 ml) was emulsified in an equal volume of FIA or FCA. These suspensions were injected intracutaneously (i.c.) at four sites over the abdomen.

Assays for delayed hypersensitivity (DH) and antibody formation. Mice were immunized intraperitoneally (i.p.) or i.c. with 2×10^7 SRBC alone, mixed with various numbers of lactobacilli, FIA, FCA or lactobacilli in FIA.

After various intervals the mice were injected subcutaneously (s.c.) in the left hind footpad with $1{\cdot}25\times10^8$ SRBC in 50 µl saline. The footpad swelling 24 hr after injection was measured with a semi-electronic pawmeter (Van Dijk, Versteeg & Hennink, 1976). This was used as a measure for delayed hypersensitivity. Reactions were recorded against the day when the test dose of antigen was injected, rather than the day upon which the reaction was measured.

On different days after immunization the numbers of direct anti-SRBC antibody-forming cells (PFC) in the spleens were determined by the plaque technique of Jerne & Nordin (1963).

Granuloma formation. Groups of mice were injected subcutaneously with 10^8 viable or heat-killed *L. plantarum* in 50 µl saline. Day 3, 4 and 5 the injected skin pieces were removed. Paraffin-embedded sections were stained and examined histologically by the Dr A.A. van den Broek, Laboratory for Histology, State University of Groningen, The Netherlands.

Statistical analysis. Results have been expressed as the arithmetic mean (m) of n (number of observations) values± standard error of the mean (s.e.m.). The significance of the results was analysed by performing a one-tailed Student's t-test. P values greater than 0·05 were considered not significant (n.s.).

RESULTS

Adjuvanticity of lactobacilli by the intracutaneous route

Mice were immunized i.c. with 2×10^7 SRBC with deep-frozen lactobacilli. At days 3 or 5 the mice were tested and the footpad swelling was measured 24 hr later. Fig. 1 shows that *L. plantarum* had significant adjuvant properties for delayed hypersensitivity to SRBC on both days tested, whereas *L. brevis* had not. No haemagglutinating antibodies against SRBC could be demonstrated in the sera of these mice (data not given).

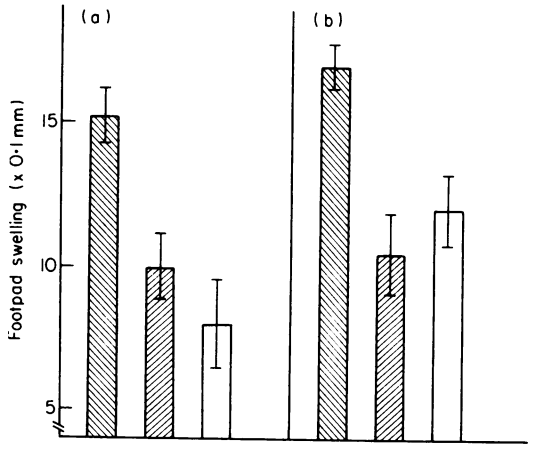

FIG. 1. Adjuvant effect on delayed hypersensitivity of two lactobacillus species. 2×10^8 of deep-frozen *L. plantarum* (▨) or deep-frozen *L. brevis* (▨) were mixed with 2×10^7 SRBC before i.c. immunization ($n=7$). Controls (□) received saline instead of lactobacilli. The animals were tested for delayed hypersensitivity at day 3 (a) or day 5 (b) after immunization. Bars indicate 1 s.e.m. The footpad swelling of mice injected with *L. plantarum* was significantly enhanced (a) $P=0{\cdot}0008$ and (b) $P=0{\cdot}0024$.

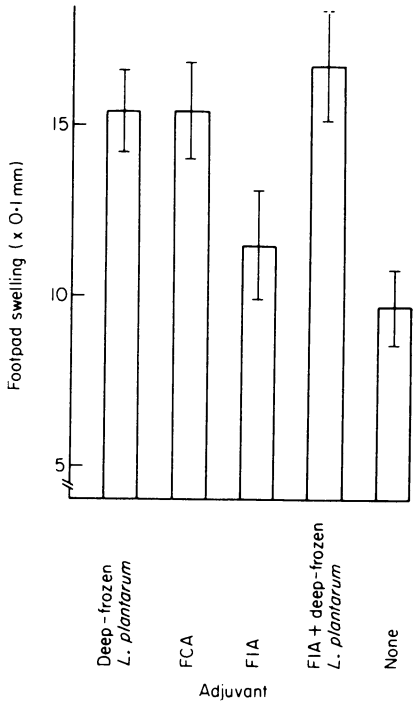

FIG. 2

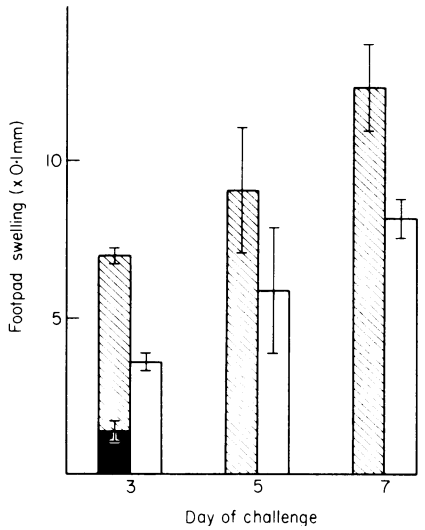

FIG. 3

FIG. 2. Comparison of the adjuvant effect on delayed hypersensitivity produced by *L. plantarum* and mineral oil adjuvants. Groups of seven mice were immunized i.c. with 2×10^7 SRBC mixed with an adjuvant or saline and challenged for delayed hypersensitivity at day 5. Bars indicate 1 s.e.m. The footpad swelling was significantly increased for deep-frozen *L. plantarum* ($P = 0{\cdot}0020$), FCA ($P = 0{\cdot}0042$) and a mixture of *L. plantarum* and FIA ($P = 0{\cdot}0040$).

FIG. 3. Time dependency of the adjuvant effect of *L. plantarum* on delayed hypersensitivity to SRBC. Groups of mice ($n = 7$) were immunized i.p. with 2×10^7 SRBC mixed with 2×10^8 of deep-frozen *L. plantarum* (▧) or saline (□). One control group was only injected with lactobacilli (■). Bars indicate 1 s.e.m. On day 3, 5 or 7 the animals were elicited for delayed hypersensitivity. The 24 hr footpad swelling of *L. plantarum* injected and immunized mice was significantly enhanced on day 3 ($P< 0{\cdot}0001$) and day 7 ($P = 0{\cdot}0076$).

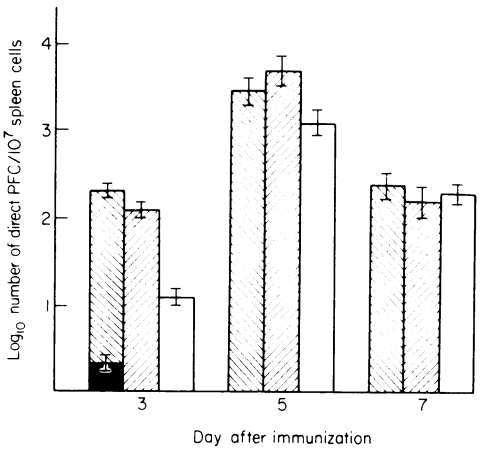

FIG. 4. Adjuvant effect on plaque-forming cell response of two lactobacillus species. Groups of mice ($n=5$) were immunized i.p. with 2×10^7 SRBC, mixed with 2×10^8 of deep frozen *L. plantarum*-df (▧), deep frozen *L. brevis* (▨), or saline (□). One control group was only injected with *L. plantarum* (■). Bars indicate 1 s.e.m. The enhancement of the numbers of splenic PFC of the immunized mice on day 3 for both lactobacillus species ($P<0{\cdot}0001$) and on day 5 for deep frozen *L. brevis* ($P=0{\cdot}0210$) was significant.

In following experiments the adjuvant activities of deep-frozen *L. plantarum*, FCA, FIA and deep-frozen *L. plantarum* suspended in FIA were compared (Fig. 2). The adjuvanticity of *L. plantarum* equalled that of FCA. The addition of FIA to the lacobacilli failed to increase the adjuvanticity significantly. The adjuvant effect of FIA on delayed hypersensitivity was not significant.

Stimulation of both cellular and humoral response after i.p. immunization

Intraperitoneal injection of mice with 2×10^7 SRBC results in both cellular and humoral immune responses (Kerckhaert, 1974).

Mice were immunized i.p. with 2×10^7 SRBC with or without 2×10^8 deep-frozen *L. plantarum*. At days 3, 5 or 7 the animals were injected with a test dose of SRBC and 24 hr later delayed hypersensitivity was measured. From Fig. 3 it can be seen that i.p. injection of *L. plantarum* enhanced delayed hypersensitivity to SRBC at all the intervals studied. No non-specific induction of delayed hypersensitivity by *L. plantarum* alone was found.

For the antibody response mice were immunized i.p. with 2×10^7 SRBC with or without 2×10^8 deep-frozen *L. plantarum* or *L. brevis*. Three, 5 or 7 days later the numbers of direct anti-SRBC PFC in the spleens of these animals were determined (Fig. 4). Both *L. plantarum* and *L. brevis* stimulated the antibody response on days 3 and 5, but no longer on day 7. The increase of the response on day 3 by *L. plantarum* amounted to twenty times the control value. Only background numbers of anti-SRBC PFC were found after injection with lactobacilli alone. In further experiments only *L. plantarum* was used.

Differential adjuvant effects of live and dead bacteria

Living *Mycobacterium tuberculosis*, injected into the peritoneal cavity of guinea-pigs, enhanced antibody formation to various antigen introduced by the same route, whereas killed mycobacteria were almost ineffective (reviewed by Freund, 1947). As deep-frozen *L. plantarum* contains both viable and dead organisms, freshly cultured and heat-killed *L. plantarum* was used for further experiments. Mice were immunized i.p. with 2×10^7 SRBC in saline or mixed with suspensions of increasing concentrations of viable or killed *L. plantarum*. At day 3 the animals were tested for delayed hypersensitivity. As shown in Fig. 5a, viable bacteria increasingly stimulated delayed hypersensitivity up to a dose of 10^8. No stimulation of delayed hypersensitivity could be observed using killed bacteria.

For the antibody response, the numbers of direct PFC were determined in the spleens of mice immunized similarly on day 3. In this instance the killed bacteria showed adjuvant activity with an optimum at 10^8 (Fig. 5b). Viable lactobacilli did not cause enhancement of antibody formation.

Optimal administration of L. plantarum as adjuvant

L. plantarum only showed adjuvant effects when injected at the same site as the antigen. This raised the question as to whether simultaneous administration of adjuvant and antigen was needed. Therefore, 10^8 viable or killed *L. plantarum* were injected i.p. 24 or 8 hr before, simultaneously with, or 8 or 24 hr after i.p. injection of 2×10^7 SRBC. Delayed hypersensitivity was elicited or antibody formation was measured 3 days after immunization. Fig. 6a shows that the adjuvant effect of viable *L. plantarum* for delayed hypersensitivity was restricted to administration between 8 hr before and 24 hr after immunization. Early antibody formation was stimulated by killed bacteria when these were injected 8 hr before or simultaneously with SRBC (Fig. 6b).

Induction of hepato-splenomegaly by L. plantarum

Mice were injected intravenously with 10^8 viable or killed *L. plantarum*. One or 4 days later the weights of liver and spleen were determined. Both killed and viable bacteria caused an increase in liver and spleen weights on day 4 (Table 1), with a greater effect of the latter.

Granuloma formation

Subcutaneous injection of mice with viable or heat-killed *L. plantarum* resulted 3 and more days later in a clearly visible nodule. Histological examination indicated that the nodule was due to an oedematous

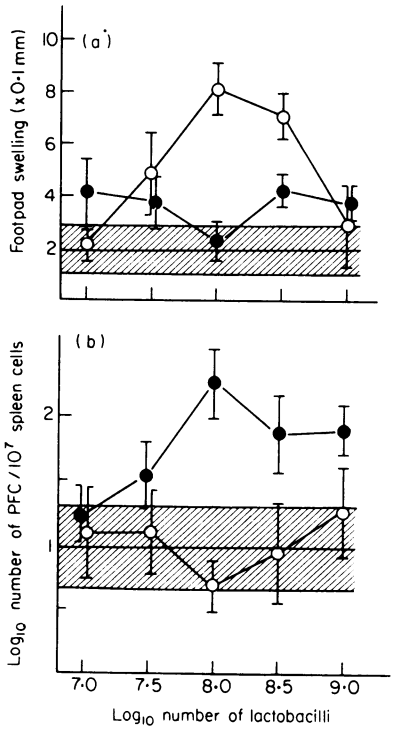

FIG. 5

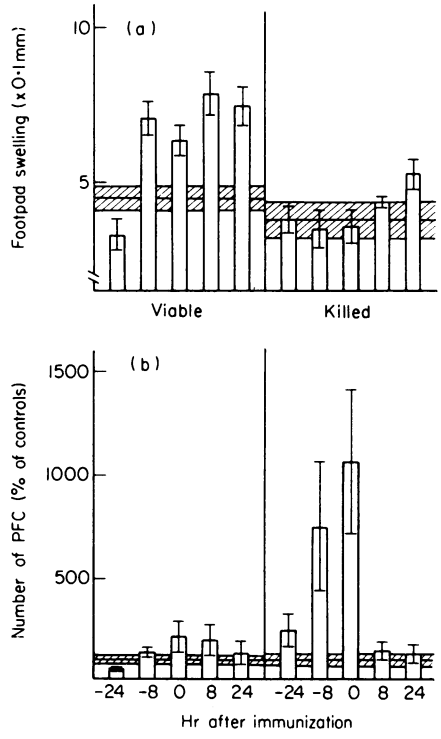

FIG. 6

FIG. 5. Differential effect of viable and killed lactobacilli. Graded numbers of viable (○) or killed (●) *L. plantarum* were injected simultaneously with i.p. injections of 2×10^7 SRBC. The delayed hypersensitivity (a) and numbers of PFC (b) were measured 3 days later. (▨) Indicates the response of animals immunized without adjuvant. Bars indicate 1 s.e.m. Footpad swelling after 10^8 and 3×10^8 viable lactobacilli was significantly enhanced ($P = 0{\cdot}0003$ and $P = 0{\cdot}0013$, respectively). Significant enhancement of numbers of PFC was found with 10^8 ($P = 0{\cdot}0026$), 3×10^8 ($P = 0{\cdot}0275$) and 10^9 ($P = 0{\cdot}0116$) killed lactobacilli.

FIG. 6. Effect on delayed hypersensitity and splenic plaque-forming cell response of an interval between the administration of lactobacilli and SRBC. Viable or killed lactobacilli were injected i.p. at different intervals before, simultaneously with or after injection with 2×10^7 SRBC. Three days later delayed hypersensitivity (a), ($n = 7$) and the humoral immune response (C), ($n = 5$) were determined. (▨) Responses of mice immunized with only SRBC. Bars indicate 1 s.e.m. Footpad swelling was significantly enhanced when viable lactobacilli were administered at −8 hr ($P = 0{\cdot}0381$), 0 hr ($P = 0{\cdot}0407$), +8 hr ($P = 0{\cdot}0202$) and +24 hr ($P = 0{\cdot}0247$). Killed lactobacilli augmented the number of PFC significantly when administratrated simultaneously with SRBC ($P = 0{\cdot}0030$).

TABLE 1. Effect of i.v. injection of viable and killed *L. plantarum* on liver and spleen weights

Injection of 10^8 *L. plantarum*	Day	Liver		Spleen	
		Weight*	*P* value†	Weight*	*P* value†
None		828±19		86±4	
Viable	−1	945±30	0·015	107±5	0·015
Viable	−4	1046±46	0·005	156±6	0·0003
Killed	−1	932±16	0·007	96±3	n.s.
Killed	−4	903±22	0·031	123±9	0·010

* In mg/20 g body weight (mean±s.e.m.).
† In comparison with the control group.

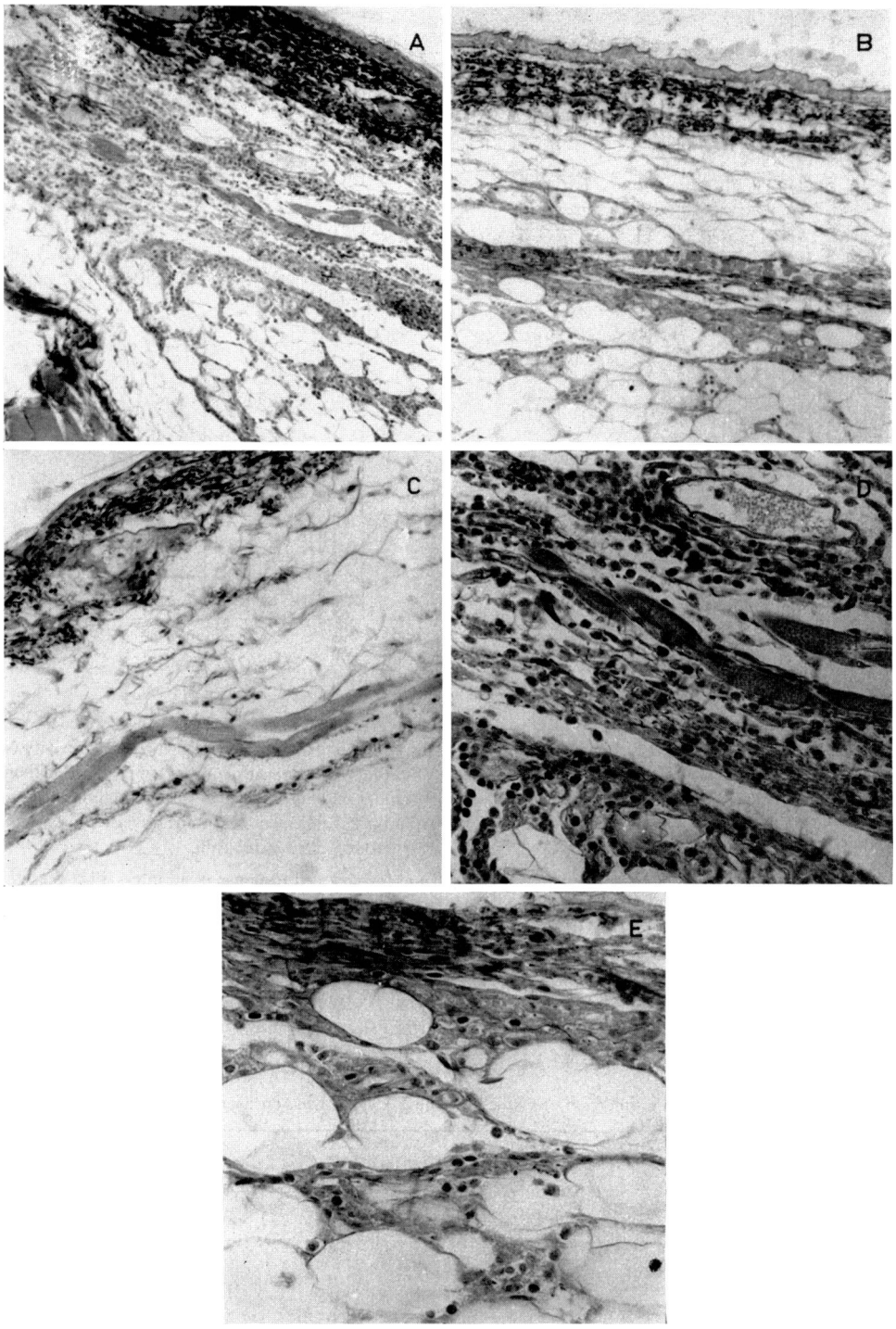

FIG. 7. Histology of granulomata developed day 5 after s.c. injection of 10^8 viable (A) (magnification × 100) and (D) (magnification × 300), or killed (B) (magnification × 100) and (E) (magnification × 300) *L. plantarum* on the abdomen. Control animals were injected s.c. with saline (C) (magnification × 100).

reaction accompanied by a moderate inflammatory infiltrate and damage to the connective tissue. The infiltrate caused by viable lactobacilli was mainly composed of mononuclear cells whereas heat-killed lactobacilli induced an infiltration of about equal numbers of granulocytes and mononuclear cells (Fig. 7).

DISCUSSION

We have shown that preparations of lactobacilli can increase the antibody and delayed hypersensitivity responses to SRBC (Figs 1, 3 & 4). *L. plantarum* was more potent than *L. brevis*. Its adjuvant effect was not due to cross-antigenicity with SRBC (Figs 3 & 4). The cellular adjuvant effect of deep-frozen *L. plantarum* failed to be increased significantly by the addition of FIA (Fig. 2), as described for mycobacteria (Freund, 1947).

The deep-frozen preparation of *L. plantarum* which contains about equal numbers of viable and non-viable organisms caused strong stimulation of the PFC response 3 days after i.p. immunization (Fig. 4). Finger, Emmerling & Büsse (1970) observed a similar result with dead *Bordetella pertussis* and SRBC. In their experiments the PFC response on day 5 was also enhanced. The effect induced by *L. plantarum* therefore suggests an acceleration rather than an augmentation of the humoral immune response. Whereas deep-frozen *L. plantarum* caused a transient augmentation of the antibody response, delayed hypersensitivity was enhanced on all days tested (Fig. 3). This suggests that different mechanisms underlie the stimulation of antibody production and delayed hypersensitivity responses by deep-frozen *L. plantarum* suspensions.

Viable and heat-killed lactobacilli were therefore tested separately. Viable bacteria stimulated only delayed hypersensitivity, whilst killed lactobacilli only augmented the antibody response (Fig. 5). The optimal number for both effects was 10^8 viable or heat-killed bacteria, corresponding to the number of 2×10^8 of the deep-frozen preparation.

In contrast, infection with viable virulent or attenuated mycobacteria is reported to be stimulatory for both antibody formation and tuberculin-type hypersensitivity to a variety of antigens in guinea-pigs, whereas stimulation is negligible or absent when killed mycobacteria were substituted for living ones (Freund, 1947). A possible explanation for the difference in adjuvanticity of living and killed mycobacteria can be found in the inhibition of phagosome–lysosome fusion by the former (Goren, 1977). Besides viable mycobacteria, a number of agents with adjuvant properties like dextran sulphate (Diamantstein *et al.*, 1971; McCarthy, Arnold & Babcock, 1977), suramin (Van der Meer, Hofhuis & Willers, 1977), concanavalin A (Lawrence & Schnell, 1977) and carrageenan (Mizushima, Murata & Horiuchi, 1974) are inhibitors of phagosome–lysosome fusion (reviewed by Goren, 1977). In this context the effects of viable and killed lactobacilli on phagosome–lysosome fusion and other macrophage functions are being studied.

Hepato-splenomegaly is a common feature of immuno-stimulating agents, suggesting stimulation of the reticuloendothelial system (RES) (Mazurek *et al.*, 1976; Franzl & McMaster, 1968). Viable lactobacilli induced greater hepato-splenomegaly than heat-killed bacteria (Table 1).

Granuloma formation following the injection of microorganisms is well known (for review see Boros, 1978). Although both viable and heat-killed lactobacilli caused granuloma formation, the cellular components were different. Viable lactobacilli caused a mainly mononuclear infiltrate in contrast to the mixed granulocytic/mononuclear infiltrate which followed the injection of heat-killed lactobacilli (Fig. 7). This difference and that found for the stimulation of the RES could possibly relate to the different adjuvant effects of viable and heat-killed lactobacilli.

Stimulation of immune responses by adjuvants depends on the interval between the administration of adjuvant and antigen (Franzl & McMaster, 1968; Murgo & Athanassiades, 1975; Van der Meer *et al.*, 1977; Athanassiades, 1977). Viable lacobacilli can be injected into the immunization site from 8 hr before until 24 hr after antigen and still show an adjuvant effect on delayed hypersensitivity (Fig. 6). The adjuvant effect of heat-killed lactobacilli, however, is restricted to a very short period (8 hr) preceding immunization. This again implies that different mechanisms could be involved in the stimulation of

antibody production by killed and delayed hypersensitivity by living lactobacilli respectively.

Several bacteria with adjuvant properties have been subjected to fractionation and in a number of instances the components with adjuvant activity have been isolated (reviewed by Borek, 1977). Recently Hiu (1977) isolated a lipoglycopeptide fraction with stimulating properties for both delayed hypersensitivity and antibody formation from *Mycobacterium tuberculosis*. He showed that the glycopeptide moiety of this compound stimulated delayed hypersensitivity and the lipid moiety antibody formation. In this respect it should be mentioned that peptidoglycan-containing cell wall fractions of *L. plantarum* were reported to act as adjuvants for delayed hypersensitivity in rats and guinea-pigs, but only in combination with FIA (Kotani *et al.*, 1975; Kohashi *et al.*, 1977). The adjuvant properties of fractions of our *L. plantarum* strain have yet to be studied.

This study clearly demonstrates that suspensions of lactobacilli in saline can act as adjuvant without needing mineral oil vehicles. The augmentation of the cellular immune response by viable lactobacilli suggests that these bacteria might be useful tools for immunotherapy.

This work was supported by a grant of the Foundation for Fundamental Immunological Cancer Research (FIK), subsidized by the verein für Krebsforsschung, Arlesheim, Switzerland.

The authors are indebted to Drs J.H. Anschütz, D.A. Houwert and J.A.M. Kerckhaert for their constructive criticism during the experiments and to Petra Schmiermann for performing some final experiments.

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