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Delayed-type hypersensitivity and acquired cellular resistance in mice immunized with killed *Listeria monocytogenes* and adjuvants

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Summary. Delayed-type hypersensitivity (DH) and acquired cellular resistance (ARC) to *Listeria monocytogenes* in mice was studied following immunization with killed bacteria in combination with Freund's complete adjuvant or the adjuvant dimethyldioctadecylammonium bromide (DDA). Intracutaneous or intraperitoneal injections of killed listeria mixed with Freund's complete adjuvant did neither result in DH nor in ACR. Intracutaneous injections of killed listeria and DDA resulted in an antigen-dose dependent DH but not in ACR. Intraperitoneal injection of listeria and DDA, however, induced ACR but no DH. Optimal conditions for the induction of ACR were simultaneous intraperitoneal injection of 15 mg DDA/kg body weight and 10^7 or 10^8 listeria. The optimal interval between immunization and challenge was 7 days. No protection was found against challenge with a lethal dose of *Salmonella enteritidis*, suggesting that the protection is specific. Intraperitoneal injection of mice with DDA resulted in inhibition of phagosome–lysosome fusion in macrophages harvested 24 h later. Interference with macrophage activity is discussed as one of the possible mechanisms for the adjuvant effect of DDA.

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INTRODUCTION

Immunity in mice infected with the facultative intracellular parasite *Listeria monocytogenes* is mediated by an acquired population of specifically sensitized thymus-derived (T) lymphocytes (Blanden & Langman, 1972). These T cells function to activate macrophages, which in turn express immunity (North, 1974). Attempts to prevent listeria infection by active immunization have led to conflicting results. Vaccines composed of sublethal numbers of viable *L. monocytogenes* have been successful (Hasenclever & Karakawa, 1957; Kerckhaert, Hofhuis & Willers, 1977), but these vaccines sometimes killed experimental animals (Osebold, Njoku-Obio & Abare, 1959; Kerckhaert *et al.*, 1977). Vaccines of killed listeria are not effective (Mackaness, 1962) unless lipopolysaccharide (Rodriguez, McClatchy & Campbell, 1974) or polyanions such as suramin or dextran sulphate (Van der Meer, Hofhuis & Willers, 1977) are added. In the latter publication it was suggested that the difference in processing of viable and dead micro-organisms by macrophages might explain the induction of resistance by vaccines of live and not of killed bacteria. Impairing macrophage activity by the cytotoxic agent lipopolysaccharide (Kessel & Braun, 1965) or by inhibitors of phagosome–lysosome fusion such as suramin and dextran sulphate (Goren, Brok & Schaefer, 1974; D'Arcy Hart & Young, 1975) might result in a processing of

dead listeria by macrophages advantageous for the induction of resistance.

Dextran sulphate has also been described as an adjuvant for antibody formation (Diamantstein, Wagner, Beyse, Odenwald & Schulz, 1971) and delayed-type hypersensitivity (DH) (McCarthy, Arnold & Babcock, 1977). Both DH and acquired cellular resistance (ACR) are believed to be manifestations of cell-mediated immunity since they can be passively transferred only with viable lymphoid cells (Mackaness, 1969) and not with serum (Mackaness, 1962). It is controversial, however, whether there is a correlation between the two phenomena (Youmans, 1975; Lefford, 1975).

In this study the effects of two other adjuvants on DH and ACR to listeria are investigated. For this purpose Freund's complete adjuvant (FCA) which potentiates both humoral and cellular responses (Freund, 1956) and dimethyldioctadecylammonium bromide (DDA) a potent adjuvant for the cellular response (Dailey & Hunter, 1974; Snippe, Belder & Willers, 1977) are used.

MATERIALS AND METHODS

Animals

F_1 mice (♂ Balb/c × ♀ Swiss inbred) were bred and maintained in the Laboratory of Microbiology, Utrecht, The Netherlands. Female mice were used at an age of about 11 weeks (20 g).

Bacteria

Listeria monocytogenes, strain L 347, serotype IV B was used in all experiments. The bacteria were grown in Brain-Heart Infusion broth (Difco Laboratories, Inc., Detroit, Michigan) containing 0·3% glucose, for 16 h at 37°, centrifuged at 12,000 *g* for 1 h, washed and resuspended in saline. They were frozen in small aliquots at −70°. *Salmonella enteritides*, strain R.I.V. 74-11264, of the National Institute of Public Health, Bilthoven, The Netherlands, was used for specificity control. The procedure for growing and storing was as described for *L. monocytogenes*.

Adjuvants and immunization

Freund's complete adjuvant (FCA, Difco Laboratories, Inc., Detroit, Michigan, containing *Mycobacterium butyricum)* and dimethyldioctadecylammonium bromide (DDA, Eastman Kodak Co, Rochester, N.Y.), were used as adjuvants. The latter was freshly suspended in saline to the appropriate concentrations.

Mice were intracutaneously (i.c.) immunized with heat-killed (56° for 1 h) listeria suspended in 0·05 ml saline mixed with an equal volume of adjuvant. For intraperitoneal (i.p.) immunization heat-killed listeria suspended in 0·25 ml saline and mixed with an equal volume of adjuvant were used.

Assay for delayed-type hypersensitivity

Delayed-type hypersensitivity was measured as the increase in footpad thickness (Kerckhaert, Van den Berg & Willers, 1974) 24 h after injecting an eliciting dose of 5 μg of lyophilized disrupted listeria, suspended in 50 μl saline; the bacteria were mechanically disrupted with a Hughes press (Sagers, 1962). Measurements were made with a semi-automatic footpad-meter (Van Dijk, Versteeg & Hennink, 1976). Reactions are recorded against the day when the eliciting injection was given, rather than the day upon which the reaction was measured.

Determination of acquired cellular resistance

The 14-day mean lethal dose (LD_{50}) of viable *L. monocytogenes* in F_1 mice was found to be 2×10^5 colony-forming units after i.p. injection. The LD_{50} values were calculated by the method of Reed & Muench (1938). Mice were i.p. challenged with 50 LD_{50} of *L. monocytogenes* and the numbers of survivors 2 weeks later were recorded. Due to the great virulence of *Salmonella enteritides* it was not possible to determine the LD_{50} for this micro-organism. When normal mice were injected with 50–100 viable *S. enteritides* all mice died within 2 weeks.

Assay for phagosome–lysosome fusion

Tests for the observation of phagosome–lysosome fusion in macrophages *in vitro* were performed as described by Goren, D'Arcy Hart, Young & Armstrong (1976). As DDA containing suspensions are turbid, the effect of DDA on phagosome–lysosome fusion was studied on macrophages harvested 24 h after i.p. injection of 15 mg DDA/kg body weight.

Statistical analysis

Results are expressed as the arithmetic mean of *n* independent observations ± standard error of the mean.

RESULTS

DH and ACR following injection of dead listeria mixed with adjuvants

Intracutaneous or intraperitoneal injections of 10^5, 10^6, 10^7 or 10^8 heat-killed listeria with or without FCA did not result in DH or ACR. Neither was this result affected by increasing the amount of mycobacteria in FCA from 0·5 to 1·5 mg/ml, or by varying the intervals between immunizing and eliciting or challenge injections.

In the following experiments DDA was used as adjuvant. Intracutaneous injection of killed listeria with 5 mg DDA/kg resulted in DH with doses of 10^4–10^6 listeria (Fig. 1). Optimal DH was measured 7–10 days after immunization with 10^5 listeria in DDA. None of the i.c. immunized mice, however, was protected against challenge with 50 LD_{50}.

Intraperitoneal injection of killed listeria mixed with DDA resulted in ACR (Table 1) but not in DH. The degree of protection depended on the dose of DDA: 15 mg/kg being optimal. With this dose of DDA 10^7 or 10^8 killed listeria afforded complete protection while even the small immunizing dose of 10^6 killed listeria was sufficient to protect four out of six mice.

Table 1. Induction of resistance by killed listeria and DDA

Immunization dose	mg DDA/kg body weight				
	50	15	5	1·5	None
10^8	n.d.	6/6	5/6	1/6	0/6
10^7	4/6	6/6	2/6	0/6	0/6
10^6	3/6	4/6	3/6	1/6	0/6
10^5	0/6	0/6	1/6	1/6	0/6
None	0/6	0/6	0/6	0/6	0/6

Mice received a challenge dose of 50 LD_{50} seven days after immunization. n.d., not done. The numbers of survivors on the numbers of mice challenged are given.

Effect of the interval between i.p. injections of DDA and killed listeria on ACR

Groups of mice were i.p. injected with 15 mg DDA/kg 48 or 8 h before, simultaneous with, or 48 h after i.p. immunization with 10^7 killed listeria. Only simultaneous injection of DDA and killed listeria resulted in complete protection (Fig. 2). Partial protection was obtained when DDA preceded the injection of listeria. No protection was found when DDA was injected

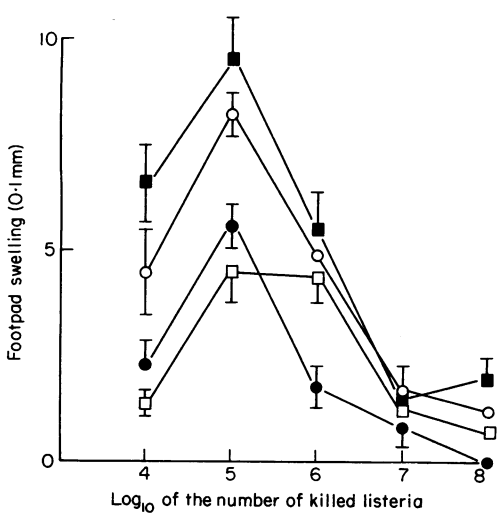

Figure 1. Effect of DDA as adjuvant on DH following intracutaneous injection of killed listeria. Groups of six mice were i.c. injected with graded numbers of killed listeria in 5 mg DDA/kg and after 5 (●), 7 (○), 10 (■) or 17 days (□) tested for DH. Vertical bars give the standard error of the mean.

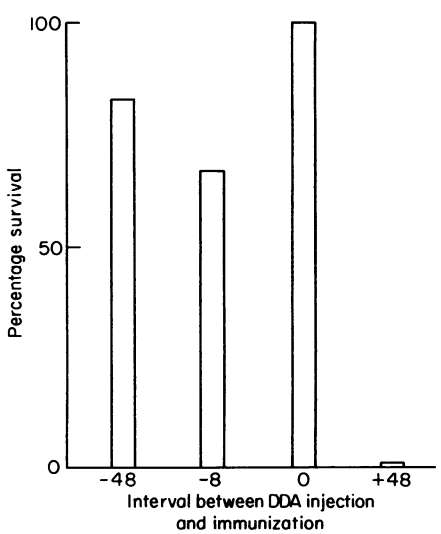

Figure 2. Effect on ACR of variation in the interval between listeria and DDA injections. Groups of six mice were i.p. injected with 15 mg DDA/kg 48 h or 8 h before, simultaneously with or 48 h after i.p. immunization with 10^7 killed listeria. Seven days after immunization all animals were i.p. challenged with 50 LD_{50}.

after the listeria. In further experiments listeria was injected mixed with DDA.

Duration of ACR induced by DDA and killed listeria

Groups of mice were i.p. injected with 10^5, 10^6 or 10^7 killed listeria mixed with 15 mg DDA/kg. At various intervals after immunization the animals were challenged with 50 LD_{50}. From Fig. 3 it is clear that following immunization with 10^7 listeria complete protection was found at day 7, whereas hardly any protection was found at day 10. On the one hand the protection following immunization with 10^6 bacteria was never complete (maximal: 80% at day 5) but on the other hand it decreased more gradually, resulting in a still 50% protection at day 10.

Specificity of ACR

In order to study the specificity of the ACR cross infection with the non-related bacterium *S. enteritidis* was studied. Two groups of mice were i.p. immunized with 10^8 killed listeria mixed with 15 mg DDA/kg. Seven days later one group was challenged with 50 LD_{50} listeria, the other group with 100 salmonellae. The mice were completely protected against listeria (Fig. 4). No protection against *S. enteritidis* was found.

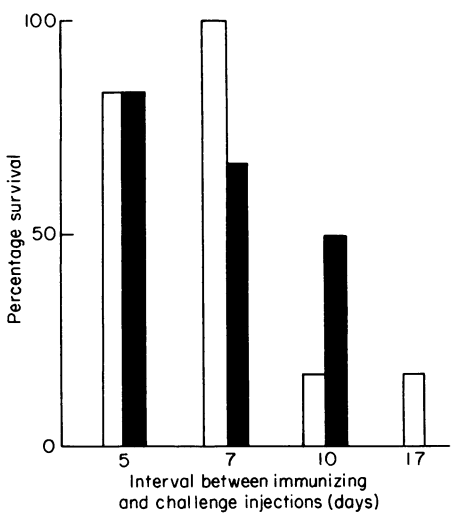

Figure 3. Duration of resistance following injection of killed listeria and DDA. Groups of six mice were i.p. injected with 10^6 (black columns) or 10^7 (white columns) killed listeria mixed with 15 mg DDA/kg. At various intervals after the immunization the animals were challenged with 50 LD_{50}.

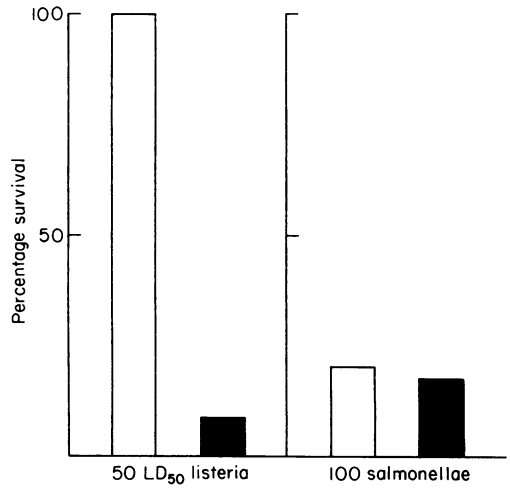

Figure 4. Specificity of resistance following injection of killed listeria and DDA. Groups of mice were i.p. injected with 10^8 killed listeria mixed with 15 mg DDA/kg (open columns, $n=20$) or 10^8 killed listeria in saline (filled columns, $n=12$). Seven days after immunization one group was i.p. challenged with 50 LD_{50} listeria and the other group with approximately 100 *Salmonella enteritidis*.

Effect of DDA on phagosome–lysosome fusion

As the effect of DDA on ACR to listeria infection is the same as that of suramin and dextran sulphate (Van der Meer *et al.*, 1977) the influence of DDA on phagosome–lysosome fusion was studied. Mice were i.p. injected with 15 mg DDA/kg or with saline. The mice were killed after 24 h and the peritoneal cells collected and tested for phagosome–lysosome fusion. Normal fusion was found in macrophages of saline treated mice but the fusion was completely inhibited in macrophages of DDA treated mice.

DISCUSSION

Intracutaneous injection of viable listeria results in DH and ACR (Kerckhaert *et al.*, 1977). Induction of resistance by a single injection of killed listeria was shown to be effective in combination with the polyanions suramin or dextran sulphate (Van der Meer *et al.*, 1977), of which the latter has recognized adjuvant activities (Diamantstein *et al.*, 1971; McCarthy *et al.*, 1977).

In the present study no DH or ACR could be induced with killed listeria in combination with FCA. Neither increasing the mycobacterium content of

FCA nor changing the conditions of administration of antigen or adjuvant resulted in a cellular response. With the adjuvant DDA an antigen-dose dependent DH was obtained with optima at 7–10 days after i.c. injection of 10^5 killed listeria. Under the conditions tested no DH could be induced via the i.p. route. On the other hand this was the route of choice to induce ACR. Complete protection was obtained with 10^7 and 10^8 killed listeria mixed with 15 mg/kg. Combinations of DDA (5 mg/kg) and killed listeria (10^7 and 10^8) which did not induce DH when i.c. injected, resulted in partial protection upon i.p. injection. ACR could not be induced via the i.c. route with any of the combinations of DDA and killed listeria. The induction of DH with DDA and listeria if i.c. injected and the induction of ACR if i.p. injected support a dissociation between both manifestations of cell mediated immunity as already suggested by others (Lefford, 1975; Youmans, 1975; Kerckhaert *et al.*, 1977).

A prerequisite for the understanding of the mode of action of immunological adjuvants would seem to be the determination of which of the cells involved in the immune response are affected by them. The necessity to inject DDA and listeria via the same route and preferentially in a mixture suggests that DDA interferes with the local antigen handling in which phagocytes are the most likely involved cells. The observation that DDA inhibited phagosome–lysosome fusion supports our hypothesis that hampered antigen processing by macrophages favours the induction of ACR. Upon immunization with viable listeria the intracellular growing bacteria inhibit optimal macrophage function. This will result in incomplete processing of listeria and sparing of determinants essential for the induction of ACR. Although impairment of macrophage function seems to be a prerequisite for the induction of ACR both by viable listeria and by killed listeria in combination with DDA or polyanions it should be stressed that no conclusions from our experiments with killed listeria can be drawn about the mechanisms used by viable listeria to evade killing by macrophages.

The mode of action of DDA has been extensively studied using hapten-carrier complexes as antigen and DH as parameter. Dailey & Hunter (1974) thought the lipid groups of DDA to be responsible for the preferential induction of cellular responses by DDA–antigen mixtures. Introduction of hydrophobic groups in an antigen promotes the localization of antigen in the paracortex of lymph nodes after i.c. immunization (Coon & Hunter, 1973). This might be an explanation for the induction of DH following i.c. immunization with listeria in DDA. Snippe, Johannesen, Inman & Merchant (1978) found a relative lack of specificity in DH upon immunization with antigen in DDA. According to these authors the specificity reducing effect of DDA might be induced by strengthening the binding of antigen–DDA complexes to T-cell receptors on the basis of their ability to bind by non-specific hydrophobic forces to T-cell membrane lipid. Thus weak interactions between a hapten and any receptor even partially specific for it are stabilized.

Based on our results and those of other authors the adjuvant effect of DDA for cellular responses might be explained by at least three possibly additionally operating mechanisms, (1) impairment of antigen processing by macrophages, (2) localization of the antigen in the T-dependent areas of the lymphoid organs and (3) stabilizing of the interaction between antigen and T cell.

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