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Impaired macrophage functions as a possible basis of immunomodification by microbial agents, tilorone and dimethyldioctadecylammonium bromide

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Four microbial and two chemically defined immunomodulating agents namely viable BCG, killed *Mycobacterium butyricum*, killed *Lactobacillus plantarum*, zymosan, tilorone, and dimethyldioctadecylammonium bromide (DDA) were studied for their effects on macrophage functions *in vitro* and *in vivo*. All agents induced a dose-dependent mortality of macrophages as determined by trypan blue exclusion. DDA and especially tilorone were rather toxic for macrophages *in vitro*. All agents except tilorone and DDA inhibited phagocytosis of yeast cells and uptake of acridine orange *in vitro* at doses which killed up to about 30% of the macrophages. DDA and tilorone had no effect at similar doses. All agents but zymosan inhibited the spreading of macrophages. No interference with the fusion of lysosomes and yeast cell-containing phagosomes could be observed. The activity of the mononuclear phagocytic system (MPS) *in vivo* as measured by carbon clearance was stimulated by all substances within twenty-four hours. All agents but DDA and tilorone enhanced non-specific bacterial resistance. As demonstrated previously for DDA, tilorone could serve as adjuvant for induction of specific resistance to *Listeria monocytogenes*.

The results are discussed in relation to other data on influencing of macrophage functions and on immunomodification. It is concluded that hampered antigen destruction by local macrophage suppression attended with MPS stimulation might be a basic mechanism for adjuvanticity exerted by these agents.

INTRODUCTION

In a previous paper (Bloksma et al., 1980) we described the effects of the polyanions carrageenan, dextran sulphate, polyanetholesulphonate and suramin on macrophage functions in mice in order to get a better understanding of their immunomodulating properties. The polyanions enhanced phagocytosis of yeast cells *in vitro* and all polyanions but carrageenan inhibited phagosome-lysosome fusion. The function of the mononuclear phagocytic system (MPS) as measured by carbon clearance, was strongly inhibited shortly after intraperitoneal administration of polyanions. In some instances this was followed within seventy-two hours by a stimulation. These results are consistent with earlier data (Van der Meer et al., 1977; Arora and Crowle, 1978) which suggest that immunomodification may be the result of altered antigen processing as a consequence of drug-macrophage interaction.

In this paper we report the effects of several immunomodifying agents of microbial or synthetic origin on the above-mentioned parameters. Moreover their efficacy to induce non-specific and, when possible, specific resistance to a lethal challenge with *Listeria monocytogenes* has been examined.

MATERIALS AND METHODS

Immunomodifying agents

The following agents were tested: viable BCG (Immun BCG Pasteur F, Institut Pasteur, Paris, France), dimethyldioctadecylammonium bromide (DDA, Eastman Kodak Company, Rochester, NY, USA), lyophilized killed *Lactobacillus plantarum,* lyophilized killed *Mycobacterium butyricum* (Difco Laboratories Inc., Detroit, Michigan, USA), tilorone-HCl (kindly provided by Dr W. L. Albrecht, Merrell National Laboratories, Division of Richardson-Merrell, Inc., Cincinnati, OH, USA), and zymosan (Gist-Brocades, Delft, The Netherlands). *L. plantarum* was grown under anaerobic conditions in DeMan-Rogosa-Sharpe broth (Oxoid Ltd, London) at 37 °C for 16 hours. Bacteria were washed three times with saline, killed by heat (1 hour at 56 °C) and lyophilized.

The agents were dissolved or suspended in 200 µl Eagle's minimal essential medium (MEM, Flow Laboratories, UK) for the *in vitro* experiments and in 0.5 ml saline for the *in vivo* experiments. DDA, zymosan, and lyophilized bacteria were sonicated for 10 seconds with a Branson sonicator B12 before use. *In vitro* all agents were tested in doses ranging from 1–300 µg/ml.

Mice

F_1 (Swiss ♀ × BALB/c ♂) mice were bred and maintained in our own facilities. Male mice were used at an age of about twelve weeks.

Test bacteria

Listeria monocytogenes (strain L347, serotype IV B) was grown in Brain Heart Infusion broth (BHI; Difco Laboratories Inc., Detroit, Michigan, USA) containing 0.3% glucose, at 37 °C for 16 hours and washed by centrifugation at 12000 × *g* for 1 hour in saline. Suspensions in saline were frozen in small aliquots at −70 °C. The dose which killed 50% of male F_1 mice within 14 days after i.p. administration (LD_{50}), as calculated by the method of Reed and Muench (1938), was 2×10^5 colony-forming units (CFU). For immunization a portion of the bacteria was killed by heating at 56 °C for 1 hour. A check for sterility on BHI and blood agar plates incubated at 37 °C for 48 hours indicated that all bacteria had been killed.

Salmonella enteritidis (strain R.I.V. 74-112664, obtained from the National Institute of Public Health, Bilthoven, The Netherlands) was grown and stored as described for *L. monocytogenes*. Due to the high virulence the LD_{50} could not be determined, but i.p. injection of 50–100 CFU into untreated F_1 mice was always lethal to all mice within 2 weeks.

Macrophage functions in vitro

Macrophage functions *in vitro* were studied according to Hart and Young (1975). In brief: Unelicited peritoneal cells were harvested in supplemented MEM (Eagle's minimal essential medium supplemented with heat-inactivated foetal calf serum, penicillin, streptomycin, amino acids, sodium pyruvate and 2-mercaptoethanol), washed and diluted to 1×10^6 cells per ml. Two ml of this suspension were incubated in Leighton tubes with glass cover slips at 37 °C in 5% CO_2 in air saturated with water for 2 hours. Cover slips were carefully rinsed to remove non-adherent cells and supernatant was replaced by fresh supplemented MEM in which an immunomodifying agent had been dissolved or suspended. Cells were further incubated for 18 hours. Ten min prior to termination of the incubation acridine orange was added to label the lysosomes. Cells were washed and incubated once more for 4 hours with baker's yeast cells suspended in phosphate-buffered saline (pH 7.2) with 2.5% fresh pooled human serum.

Cover slips were then carefully rinsed and examined microscopically with blue-violet light. The following characteristics of 100 macrophages were recorded: numbers of ingested yeast cells, appearance of phagosome-lysosome fusion and extent of macrophage spreading.

Under normal conditions 95% of the viable macrophages had ingested 2–4 yeast cells. Phagocytosis was considered enhanced if they contained 5 or more yeast cells, and inhibited if less than 5% of the macrophages contained yeast cells.

Under normal conditions phagosome-lysosome fusion was present in 95% of the macrophages as judged by orange-coloured ingested yeast cells. If less than 5% of the macrophages contained orange-coloured yeast cells, phagosome-lysosome fusion was considered to be inhibited.

Table 1. Effect of microbial agents, DDA and tilorone on macrophage functions *in vitro*

Agent	Dose	Phagosome-lysosome fusion	Yeast cell phagocytosis	Acridine orange uptake by lysosomes	Macrophage spreading	Percentage of dead macrophages
	μg					%
BCG	10	○	○	○	○	19.9
	30	×	↓	○	○	29.0
	100	×	↓	↓	↓	34.9
M. butyricum	300	×	↓	↓	↓	19.6
L. plantarum	10	○	○	○	○	15.2
	30	○	○	○	○	16.0
	100	×	↓	○	↓	30.7
	300	×	↓	↓	↓	39.4
Zymosan[1]	3	○	○	○	○	8.3
	10	○	○	○	○	12.4
	30	×	↓	○	○	25.8
DDA	10	○	○	○	○	6.6
	30	○	○	○	○	32.5
	100	×	↓	↓	↓	82.7[2]
Tilorone	1	○	○	○	○	6.4
	3	○	○	○	○	27.5
	10	×	↓	↓	↓	80.0
None						4.4

○, no effect; ↓, inhibition; ×, could not be determined because of lack of phagocytosis.
[1] The numerous particles of zymosan prevented proper observation of its effect on macrophages at doses of 100 μg and higher.
[2]This figure is virtually an estimate as cell aggregation observed at this concentration prevented exact counting.

Macrophage spreading was scored according to the criteria of Rabinovitch et al. (1977). If the number of spreaded macrophages was above 25% or below 1%, spreading was considered to be enhanced or inhibited, respectively.

Viability of the macrophages was determined by trypan blue dye exclusion at the end of the incubation period with the immunomodifying agent.

All *in vitro* experiments were performed in triplicate.

Macrophage functions in vivo

Groups of 6 mice were injected i.p. with an immunomodifier and the rate of clearance of an intravenous injection of colloidal carbon from the blood was determined 4, 24 or 72 hours later (Biozzi et al., 1953). The phagocytic index was expressed as $K = (\log C_0 - \log C_t)/t$ where C_0 and C_t are the carbon concentrations in the blood at zero time and time t. The corrected phagocytic index

was expressed as $\alpha = \sqrt[3]{K}$ multiplied with the ratio of body weight and weight of liver plus spleen.

Induction of non-specific resistance to *L. monocytogenes* was measured by i.p. injection of groups of mice ($n = 6$) with an immunomodifier followed by an i.p. challenge with 15 LD_{50} viable *L. monocytogenes*. The numbers of surviving animals were recorded 14 days later. As all agents except tilorone induced considerable non-specific resistance only the latter agent was assayed for its adjuvanticity in the induction of specific acquired cellular resistance. Groups of 6 mice were immunized i.p. with tilorone and 10^8 killed *L. monocytogenes* simultaneously or at different intervals. Seven days after injection of listeria, the mice were challenged with 15 LD_{50} viable *L. monocytogenes* or 10^2 viable *S. enteritidis* to assess resistance and its specificity.

Data handling and statistics

Phagocytic indices K and α of treated animals were expressed as mean percentage in relation to the saline-treated control mice. For determination of significancy Student's t test was performed on the arithmetical means $\pm$ the standard error of the mean.

Results

Effect on in vitro activities of macrophages

All agents showed a dose-dependent toxicity as determined by trypan blue exclusion after 18 hours of incubation (Table 1). All agents except tilorone and DDA inhibited yeast cell phagocytosis at concentrations which left over 60% of the cells viable. Tilorone and DDA did not affect phagocytosis until very toxic concentrations were reached. At concentrations which did not interfere with phagocytosis all agents permitted normal fusion of lysosomes with phagosomes. Higher concentrations inhibited phagocytosis to such an extent that interference with phagolysosome formation could not be determined. Macrophage spreading was not affected by lower doses of the agents; at higher doses all agents but zymosan became inhibitory.

Effect on the in vivo clearance of carbon by the MPS

Eighteen groups of 6 mice were injected i.p. with one of the agents. Four, 24 and 72 hours later carbon was injected intravenously and clearance was measured. BCG and *M. butyricum* stimulated the phagocytic index K already 4 hours after i.p. administration while all agents did so at 24 and 48 hours after the injection (Fig. 1). The corrected phagocytic index α was significantly enhanced either 24 or 72 hours after administration of all agents but *M. butyricum*.

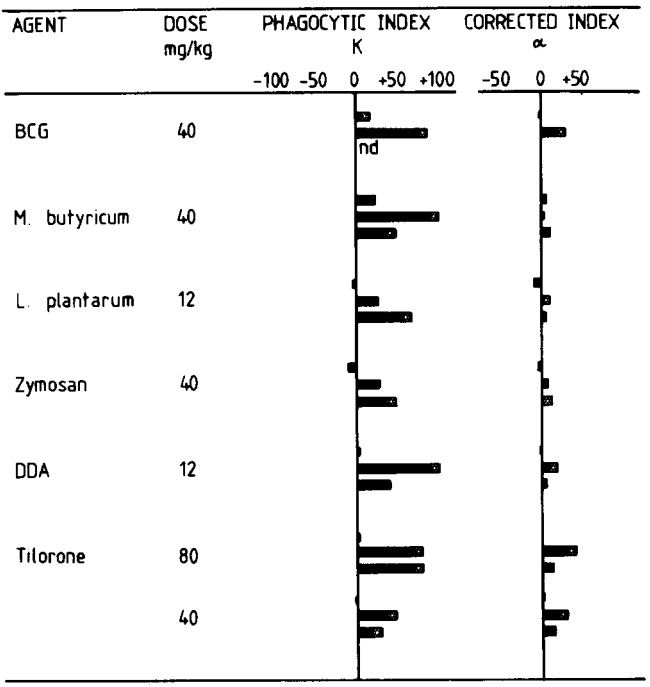

Fig. 1. Clearance of colloidal carbon and weights of spleen and liver (as % in relation to the saline-treated controls) 4, 24 and 72 hours (bars from top to bottom, respectively) after i.p. administration of microbial agents, DDA and tilorone. The doses used were selected from published data on *in vivo* immunomodification. Controls received saline.
n.d. = not done.
▬ : $p < 0.05$ in relation to saline-treated controls.

Effect on non-specific and specific resistance to Listeria monocytogenes infection

Groups of 6 or 12 mice were injected i.p. with different doses of *M. butyricum*, BCG, *L. plantarum*, zymosan, tilorone or with saline. Seven days later a challenge with 15 LD_{50} viable *L. monocytogenes* was given. Protection was afforded by injection of all agents but tilorone. The degree of protection decreased in the order given below (Table 2). As tilorone did not induce non-specific resistance, its potential as adjuvant for the induction of specific resistance could be tested. If an i.p. injection of tilorone was followed by an i.p. injection of killed *L. monocytogenes* 2 or 4 days later, part of the mice were protected against a lethal listeria infection but not against a salmonella infection (Table 3). No protection could be induced by injecting killed *L. monocytogenes* alone or mixed with tilorone.

Table 2. Induction of non-specific resistance against viable *L. monocytogenes* by immunomodifying agents

Immunomodifying agent	Dose	Protection [1]
	mg/kg	
M. butyricum	15	6/6
	5	6/6
	1.5	1/6
BCG	40	10/12
L. plantarum	40	9/12
	12	9/12
Zymosan	40	6/12
Tilorone	400	0/6
	120	0/6
Saline		0/6

[1] Immunomodifying agents and viable *L. monocytogenes* were injected i.p. with an interval of 7 days. The protection was measured as the number of survivors on the number of mice challenged after 14 days.

Table 3. Induction of specific resistance against viable *L. monocytogenes* by injections of killed listeria and tilorone

Tilorone administration i.p.		Protection after i.p. challenge with	
Dose	Day [1]	*L. monocytogenes*	*S. enteritidis*
mg/kg			
50	− 2	0/6	ND [2]
50	0	0/6	ND
100	− 4	3/6	0/6
100	− 2	5/6	0/6
100	0	0/6	ND
none		0/6	0/6

[1] Killed listeria vaccine was administered i.p. at day 0. The challenge injection was given at day 7 and the protection was measured as the number of survivors on the number of mice challenged 14 days later.

[2] Not done.

Discussion

The immunomodulating agents studied in this paper can be characterized generally by a suppressive action on phagocytosis *in vitro* (Table 1) and a stimulation of MPS activity *in vivo* (Fig. 1 and Table 2). Suppression of *in vitro* phagocytosis was observed earlier with BCG (Nathan and Terry, 1977) and DDA (Willers et al., 1979). At concentrations which did not interfere with phagocytosis none of the agents inhibited the fusion of phagosomes and lysosomes. Due to inhibition of phagocytosis and a concomitant decrease of acridine orange uptake we could not observe interference with phagosome-lysosome fusion at higher concentrations. Nevertheless fusion inhibition of lysosomes and phagosomes containing viable BCG or other mycobacteria, but not killed mycobacteria has been observed (Armstrong and Hart, 1971). The fusion inhibition may be restricted to membranes in close proximity of the viable mycobacteria.

The observed inhibition of macrophage spreading upon incubation with the majority of the agents might be a reflection of hampered macrophage function. Spreading was shown to be effected by conversion of complement factor B to Bb either by proteases or factor D (Bianco et al., 1978; Götze et al., 1978). Decreased production of factor B or its activating enzymes as a consequence of inhibition of macrophage activity might result in reduced spreading.

In vivo all agents stimulated the pinocytic activity of the MPS as measured by carbon clearance (Fig. 1). These *in vivo* effects combined with the observed *in vitro* properties of the agents conform very well to the criteria for adjuvanticity as formulated by Arora and Crowle (1978), namely a long persisting shield against antigen degradation and a simultaneous stimulation of the MPS. It may thus be part of the mechanism of adjuvant action that macrophages in direct contact with the immunomodifying agents are hampered in their function. Many adjuvants have to be administered by the same route as the antigen (Borek, 1977), which would enable local interference with antigen degradation by the macrophage. On the other hand systemic macrophage activation might prevent the induction of tolerance and favour effector functions of immunity.

In a previous paper we have studied the effects of polyanionic adjuvants on *in vitro* and *in vivo* macrophage functions (Bloksma et al., 1980). In contrast to the agents described here the polyanions dextran sulphate, carrageenan, polyanetholesulphonate and suramin stimulated phagocytosis *in vitro*, while they inhibited carbon clearance *in vivo* initially, followed by a stimulation. Impairment of antigen degradation would be effected by their capacity to inhibit lysosomal enzyme function (Crowle et al., 1977) and to inhibit phagosome-lysosome fusion (Hart and Young, 1975; Van der Meer et al., 1977).

All microbial agents used enhanced non-specific resistance to *L. monocytogenes* (Table 2). This has been attributed earlier to macrophage activation (Blanden, 1974; Kelly, 1976; Biroum-Nourjasin, 1977). Tilorone (Table 2) and DDA (Van der Meer et al., 1979) failed to induce non-specific resistance but were

shown to be good adjuvants for induction of specific resistance to listeria (Table 3; Van der Meer et al., 1979). Unfortunately adjuvanticity of the microbial agents could not be evaluated in the present system as non-specific macrophage activation would obscure acquired cellular resistance. All agents, however, have established adjuvanticity for a variety of antigens (Borek, 1977; Werner et al., 1977; Bloksma et al., 1979).

The observation that tilorone can serve as an adjuvant for a T-cell dependent immune reaction as acquired cellular resistance to listeria (Table 3) is at variance with previous characterizations of its selective adjuvant action to wit stimulation of antibody formation and suppression of T-cell dependent immune reactions (Werner et al., 1977). These studies, however, all concern administration of antigen and tilorone by different routes and sometimes chronic administration of the drug. These and our results indicate that both time and route of tilorone administration in relation to the injection of the antigen determine the outcome of immunomodulation.

In conclusion the theory that adjuvanticity might be based on hampered antigen degradation by macrophages attended with a stimulation of MPS activity seems to be validated by the results obtained. Both the polyanions and the agents tested in this paper somehow fulfil the prerequisites for adjuvanticity, although they exert quite opposite effects on some of the parameters investigated.

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