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Vaccination of Rabbits Against *Entamoeba histolytica* with Aqueous Suspensions of Trehalose-Dimycolate as the Adjuvant

ALPANA SHARMA,[1] AFROZUL HAQ,[1] SOHAIL AHMAD,[1]* AND EDGAR LEDERER[2]

Parasitic Immunology Laboratory, Department of Microbiology, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh 202001, India,[1] and Laboratoire de Biochimie, Centre National de la Recherche Scientifique, 91190 Gif-sur-Yvette, France[2]

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Rabbits were immunized with soluble *Entamoeba histolytica* antigen with an aqueous suspension of trehalose-6,6′-dimycolate used as the adjuvant. Induction of protective immunity in the immunized animals was demonstrated by enhanced humoral and cell-mediated immune responses and 100% survival after challenge. Administration of soluble antigen only failed to induce a similar degree of protective immunity. Trehalose-6,6′-dimycolate alone produced only a slight increase in nonspecific resistance.

In experimental amebiasis, systematic evaluation of antigens and adjuvants for vaccination has not yet been carried out. The functional significance of humoral and cell-mediated immunity (CMI) in invasive amebiasis also has not been sufficiently studied. There appear to be only a few reports dealing with the induction of protective immunity in experimental amoebic infections (1, 11, 19, 39).

Entamoeba histolytica, the etiological agent of amebiasis, is known to cause recurrent infections in humans. There is very little evidence to show that previously infected hosts have any resistance to reinfection. The few attempts which have been made to induce protective immunity in experimental animals have not as yet provided sufficient evidence to justify future trials of such immunization procedures on a large scale (11, 19, 39). The antigen preparations more frequently used for immunization purposes include whole and fractionated soluble portions, amoeba cell homogenates, and ribosomal and lysosomal fractions (11, 13, 19, 30, 39). Earlier work in this laboratory has shown that amoeba antigen in combination with glucan [(β-1-3)polyglucose, an extract of the cell wall of *Saccharomyces cerevisiae*] can be successfully employed for inducing protective immunity in guinea pigs and hamsters (1, 13, 40).

The present investigation describes the activity of an aqueous suspension with trehalose-6,6′-dimycolate (TDM) as the adjuvant for immunizing rabbits with a soluble *E. histolytica* antigen. Intravenous inoculation of antigen plus TDM were found to induce protective immunity, which was monitored by in vivo and in vitro methods. The results of the above tests were consistently uniform in repeated experiments.

MATERIALS AND METHODS

Antigen. Axenic cultures of *E. histolytica* NIH-200 were raised in Diamond TP-S-1 monophasic medium (8). The cultured amoebae were pooled and washed three times in normal saline. The amoeba cells sedimented by centrifugation at 850 × *g* for 10 min were prepared for antigen extraction by lysing them through freezing and thawing five times between −20 and 4°C and later subjecting them to a shearing force (16). Protein concentration was determined by the method of Lowry et al. at 700 nm (29). Previous studies in our laboratory have shown that such antigenic extracts are immunogenically active (13, 40).

Adjuvant. TDM was a commercial preparation (Choay-Chimie, Montrouge, France) containing 1 mg of TDM per ml of sterile, pyrogen-free water.

Immunization. Forty adult male albino rabbits (Central Drug Research Institute strain), each weighing 700 to 850 g, were divided into four groups of 10 animals. In group 1, the animals were inoculated with antigen plus TDM. Each animal in this group received a total of 1 mg of antigen and 2.25 mg of TDM in three weekly inoculations. TDM was administered at the rate of 1 mg/kg of body weight. Group 2 animals received a total of 1 mg of antigen alone in three doses. Each animal in control group 3 received a total of 2.25 mg of TDM, and group 4 animals were given 1.25 ml of saline in three doses. The total volume of each injection was made up to 1.25 ml with saline. The immunization schedule was completed over a period of 4 weeks, including week 3 as a rest period. All immunizations were carried out by the intravenous route.

IHA. On day 28, antibody titers were determined by the modified indirect hemagglutination (IHA) technique of Krupp (18). A 2.5% suspension of sheep erythrocytes was tanned for 20 min with tannic acid (1:100,000) at 4°C. The tanned cells were sensitized with antigen at pH 6.4 in a water bath at 37°C for 20 min. The cells were added to a two-fold dilution of the serum, and the endpoint was taken as the lowest dilution giving 50% cell agglutination. A serum was considered positive if its antibody titer was higher than 1:64.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed on day 28 by the method of Lin et al. (28) with slight modification. Polystyrene microtiter plates (flat-bottomed; Cook Laboratory, Alexandria, Va.) were coated with antigen (1:300 dilution in 0.05 M carbonate buffer, pH 9.6) and incubated at 4°C for 24 h. The plates were then washed three times with PBS/T (phosphate-buffered saline containing 0.05% Tween 20, pH 7.4). An 0.2-ml sample of the test serum serially diluted from 1:4 to 1:65,536 with PBS/T was added to each well, and the plates were incubated at 37°C for 30 min. After incubation, the plates were again washed, and 0.2 ml of anti-rabbit immunoglobulin G–alkaline phosphatase conjugate (A 8025, Sigma Chemical Co., St. Louis, Mo.; 1:1,000 dilution with PBS/T) was added and incubated for 30 min at 37°C. Later, 0.2 ml of substrate (*p*-nitrophenyl phosphate; Sigma 104-105; 1 tablet

* Corresponding author.

TABLE 1. Humoral antibody detection by IHA and ELISA

Group (treatment)	Mean titer[a] ± SD (range)		Mean A_{400}[b] ± SD (range)	P[c]
	IHA	ELISA		
1 (antigen plus TDM)	8,192 ± 4,729 (4,096–16,384)	32,768 ± 13,377 (16,384–65,536)	1.462 ± 0.132 (1.45–1.47)	<0.001
2 (antigen)	1,024 ± 591 (512–2,048)	4,096 ± 1.672 (2,048–8,192)	1.026 ± 0.198 (1.02–1.03)	<0.01
3 (TDM)	8 ± 4.6 (4–16)	32 ± 16 (16–32)	0.372 ± 0.165 (0.35–0.38)	
4 (saline)	16 ± 9.2 (8–32)	16 ± 6.5 (8–32)	0.255 ± 0.143 (0.21–0.26)	

[a] Arithmetic mean for 10 animals per group. Antibody titers below 1:64 were considered negative.
[b] The A_{400} was measured at the corresponding ELISA titer. An A_{400} of <0.4 was considered negative.
[c] Significance was calculated on the basis of Student's *t* test (group 1 versus group 3 and group 2 versus group 4).

for each 5 ml of 10% diethanolamine buffer) was added. The reaction was stopped after 30 min by adding 0.05 ml of 3 M NaOH, and the absorbance was read at 400 nm.

Skin test. Rabbits were challenged with 12 μg of antigen protein given intradermally on day 31. The reaction intensity was observed by measuring the diameter, thickness of erythma, and induration at the skin test site at 12, 24, and 48 h after antigen inoculation. Skin tissues from the reaction sites were sectioned and stained with iron hematoxylin and eosin for histological studies.

MMI test. The macrophage migration inhibition (MMI) test was performed on day 33. Peritoneal exudate cells from rabbits were collected by a procedure described earlier (7). After the last wash, the peritoneal exudate cells were suspended in RPMI 1640 medium at a concentration of 5×10^6 cells per ml. Capillaries filled with cell suspension and centrifuged at $100 \times g$ for 5 min were cut at the cell-fluid interface and placed in Mackaness migration chambers filled with RPMI 1640 medium supplemented with 10% fetal calf serum. The medium also contained 15 μg of antigen protein per ml and antibiotics (streptomycin and penicillin). The cells were allowed to migrate for 48 h at 37°C, and the areas of migration were projected, traced, and quantified by planimetry. In the control chambers, amoeba antigen was omitted. The percentage of MMI was calculated as 100 − [(area of migration with antigen/(area of migration without antigen)] × 100.

Challenge. Half of the animals in each group were challenged intrahepatically and the other half intracecally through direct inoculation of 4×10^6 pathogenic amoebae (Central Drug Research Institute strain, India) into each animal. On postchallenge day 14, one-half of the surviving animals in group 1 were sacrificed for gross examination of the viscera, and in groups 2, 3, and 4 autopsy examinations were performed as and when the animals died or showed signs of disease. The intensity of amoebic infection was determined by the weight of the liver abscess as described by Ghadirian and Meerovitch (9). The tissue from liver and cecum was later removed and stained with hematoxylin and eosin. Stained slides of the challenged tissues were used for histopathological examination.

RESULTS

Effect of TDM on humoral immune response. Rabbits immunized with a combination of antigen and TDM showed markedly increased antibody levels compared with those in animals given antigen alone (Table 1). The former showed a ca. eightfold increase in antibody level compared with the latter, as shown by both the IHA and ELISA techniques. The sera of rabbits given either TDM or saline alone were negative for antibodies.

Effect of TDM on CMI. Delayed-type hypersensitivity studies showed that animals immunized with antigen plus TDM presented a ca. 70% increase in delayed-type hypersensitivity compared with rabbits immunized with antigen alone (Table 2). Histological examinations of the skin reaction site showed characteristic perivascular cuffing with prominent mononuclear cell infiltration, with lymphocytes and few macrophages. TDM or saline alone did not produce a delayed-type hypersensitive response.

Similarly, the MMI test showed that macrophages from rabbits treated with antigen plus TDM showed an almost 71% increase in MMI compared with those from animals given antigen only (Table 2). MMI values for the macrophages of rabbits treated with TDM alone or saline only were zero.

Effect of TDM on amoebic infection. Rabbits which were treated with saline (controls) and challenged with amoebae developed frank diarrhea preceded by lassitude, listlessness, and significant loss of appetite, whereas rabbits immunized with antigen and TDM in aqueous suspension appeared normal (Table 3). The liver and cecum of these animals were devoid of any macroscopic lesions, whereas in the controls these organs showed pinpoint amoebic lesions. The entire tissue appeared inflamed and edematous. Three rabbits in the group immunized with antigen, four in the group receiving TDM alone, and five in the control group had liver abscesses.

The liver and cecum from control animals revealed ulcerative amoebic lesions accompanied by necrosis, fibrosis, and considerable tissue damage. The livers from these animals showed pinpoint lesions appearing as decolorized raised areas. Tissues from antigen-plus-TDM-immunized animals did not show such lesions or any other sign of tissue damage. The vaccinated animals, after antigen-TDM immunization, showed a distinct protective response accompanied by a marked inflammatory reaction in the liver and cecum tissues. Histological examination of liver and cecum from the protected animals revealed prominent mononuclear cell infiltration of the lymphocytes and plasma cells, but the

TABLE 2. CMI detected by skin and MMI tests[a]

Group (treatment)	Mean lesion diam (mm) ± SD	Mean induration thickness (mm) ± SD	Mean MMI (%) ± SD
1 (antigen plus TDM)	14.99 ± 0.64[b]	4.54 ± 0.65[b]	51.14 ± 2.01[b]
2 (antigen)	8.67 ± 0.62[c]	2.78 ± 0.57[c]	29.82 ± 2.98[c]
3 (TDM)	4.37 ± 1.55	1.50 ± 0.60	8.67 ± 1.84
4 (saline)	3.11 ± 1.16	1.23 ± 0.98	4.26 ± 1.53

[a] Lesion diameter and induration thickness values less than 5 and 2 mm, respectively, were considered negative. Values are the arithmetic means for 10 animals per group.
[b] $P < 0.001$ versus group 3.
[c] $P < 0.01$ versus group 4.

TABLE 3. Animal challenge studies

Group (treatment)	No. challenged/no. survived (P)[a]		Time to death (days)	Mean of liver abscess (g) ± SD (range)	% Survival
	Intrahepatic challenge	Intracecal challenge			
1 (antigen plus TDM)	5/5 (<0.001)	5/5 (<0.001)		None detected	100
2 (antigen)	3/5 (<0.05)	2/5 (<0.02)	10–11	1.56 ± 0.19 (1.2–1.9)	50
3 (TDM)	1/5	1/5	7–9	1.94 ± 0.33 (1.6–2.2)	20
4 (saline)	0/5	0/5	3–7	2.15 ± 0.2 (1.8–2.5)	0

[a] Significance was calculated by Student's *t* test for group 1 versus group 3 and for group 2 versus group 4.

histology slides of tissues from the unprotected animals clearly showed tissue invasion by the parasites, along with necrotic and fibrotic areas.

DISCUSSION

TDM, a mycobacterial glycolipid, is a well-known immunomodulator. The immunological properties of TDM, discovered by Bekierkunst and colleagues (3, 4), have been mostly observed by injecting it in the presence of oil (20, 26, 36; for reviews, see references 2, 12, and 21–25). Such TDM solutions or emulsions can, however, lead to secondary reactions, such as production of granulomas (37), especially after intravenous injection, enhancement of endotoxin lethality, etc.

More recently, however, aqueous suspensions of TDM (first described by Kato [14, 15]) have been shown to be very active immunostimulants, inducing fewer side reactions. In vivo experiments have shown that TDM suspensions in saline can protect mice against infection by *Klebsiella pneumoniae* and *Listeria monocytogenes* (35), *Babesia microti* (6), *Schistosoma mansoni* (32), and *Plasmodium berghei* (19a). Such suspensions have also been shown to enhance the antitumor activity of macrophages in vitro and in vivo (33) when injected intraperitoneally into mice; peritoneal macrophages tested 7 days afterwards showed increased cytotoxicity to mastocytoma cells in vitro (27, 41). Quite recently, Kierszenbaum et al. (17) have reported that aqueous TDM suspensions modulate mouse macrophage function in vitro by augmenting both internalization and intracellular destruction of *Trypanosoma cruzi*.

Similarly, rats injected with aqueous suspensions of TDM produce peritoneal macrophages with cytolytic activity against syngeneic tumor cells (38). Consistent with these findings are our results showing that rabbits treated with TDM alone displayed some resistance to *E. histolytica* challenge. Macrophages have been shown to be important in host resistance to hepatic amebiasis (10). In an earlier study, animals treated with BCG (bacillus Calmette-Guérin) displayed increased nonspecific resistance to *E. histolytica*, probably through activation of the mononuclear phagocyte system (1). Most likely the TDM-enhanced resistance to *E. histolytica* observed in our study is related to the ability of TDM to increase macrophage activity. The results of in vivo skin tests, read in conjunction with in vitro MMI results, amply prove enhanced CMI after antigen-plus-TDM immunization. Ortiz-Ortiz et al. (34) have detected the appearance of a CMI response through skin and MMI testing. They have shown that results of MMI tests correlate well with those of skin tests in human subjects. Later, Bray and Harris (5) also confirmed that lymphocyte proliferation and MMI tests are the two most reliable parameters for detecting mediators which are responsible for delayed-type hypersensitivity reactions. The findings of the present study further confirm that skin and MMI test results are reproducible and in perfect agreement with one another as far as CMI detection is concerned.

In addition to this, TDM given along with amoeba antigen acted as a potent adjuvant. To date there seems to be only one report showing that TDM can be used as an adjuvant in a parasite system; mice immunized with the protoplasm of disrupted *Toxoplasma gondii* tachyzoites in an oil-in-water emulsion containing TDM showed a prolonged survival time after *T. gondii* challenge (31). The results of the present study not only confirm the adjuvant effect of TDM in another parasite system, but also show that an aqueous suspension of TDM is an effective adjuvant because of its less toxic nature.

We showed that TDM markedly enhanced both the humoral and cell-mediated arms of the immune response to amoeba antigens. This is a major reason for the 100% survival of rabbits treated with the amoeba antigen-plus-TDM combination, although nonspecific immune mechanisms may also contribute to their survival.

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