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Effect of the adjuvant dimethyl dioctadecyl ammonium bromide on the humoral and cellular immune responses to encephalomyocarditis virus

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The effects of the adjuvant dimethyl dioctadecyl ammonium bromide (DDA) on the immune responses to encephalomyocarditis (EMC) virus were studied in mice. The humoral response, as measured by appearance of neutralizing antibodies, was slightly enhanced in mice immunized by the intraperitoneal route. Intracutaneously, DDA almost did not affect the humoral response but resulted in distinct enhancement of delayed type hypersensitivity (DH), as measured by the footpad swelling test.

DH to EMC virus was found to be antigen-specific and could be passively transferred to normal mice with peritoneal exudate cells from immunized mice.

Dose–response curves for DH and humoral antibody responses to EMC virus were not concordant. Low doses induced DH on day 6 without measurable circulating antibodies; high doses gave good antibody responses but suboptimal DH reactions. Immunization conferred a state of resistance to infection with virulent EMC virus. Protection seemed more related to DH than to the prevalence of specific antibodies at the time of infection.

encephalomyocarditis virus; dimethyl dioctadecyl ammonium bromide; adjuvant; antibody; delayed type hypersensitivity

Introduction

To our knowledge, there are no literature data describing delayed type hypersensitivity (DH) or cell-mediated immunity (CMI) to EMC virus. This virus belongs to the group of the picornaviridae, which have a single-stranded RNA genome in a small eicosahedral capsid [16]. EMC virions contain neither lipids nor glycoproteins, as can be expected of nonenveloped (naked) viruses [4]. Infections with enveloped, but not with naked viruses are associated with unequivocal DH or CMI [2, 17]. The absence

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(or poor detectability) of CMI reactions to picornaviruses may be partly accounted for by the pure proteinaceous nature (no glycosylation) of their capsids. It is indeed notoriously difficult to induce DH to large unglycosylated proteins in mice [6]. Moreover, during infections with picornaviruses the host cell membrane is not changed by the insertion of virus-coded proteins. Therefore, the infected cell may not be recognized as foreign by the immune system, explaining why cytotoxic T lymphocytes do not seem to be involved in the recovery from picornavirus infection [2]. On the other hand, neutralizing antibodies appear to be of paramount importance in the eradication of infectious virus from the body as suggested by animal experiments and by the high incidence of severe infections (e.g. paralytic poliomyelitis) in humans with humoral immune deficiencies [1,15,24]. However, the available evidence does not exclude that CMI may play an accessory role in recovery from picornavirus infection. If on immunization picornavirus-specific T cells would be induced they might activate macrophages and produce interferon-γ (formerly immune interferon) [14]. This could contribute to a diminished virus replication especially in the initial phase of the infection when only minute amounts of antibodies are present [14].

The purpose of this study was to establish whether a specific T-cell reaction to EMC virus can be evoked in mice in the absence of detectable antibodies in serum and whether such mice are protected against virulent EMC virus. To enhance CMI to EMC virus the lipophilic quarternary amine, DDA, was used as an adjuvant.

Materials and methods

Virus strains

A virulent strain of EMC virus [3] was obtained from Dr. W.J.C. Bogaerts (Medical Biological Laboratory TNO, Rijswijk, The Netherlands). The subcutaneous 50% lethal dose (LD_{50}) for male BALB/c mice was 15 plaque-forming units (PFU). A large plaque variant of the virulent strain was used for plaque reduction tests and the production of inactivated EMC virus.

The avirulent Semliki Forest virus (SFV) strain MRS MP 192/7 [9] was received from Dr. K.G. Oei (Royal Tropical Institute of Amsterdam, The Netherlands) and used for control experiments.

Cells and media

Throughout this study L cells cultured in the medium of Yamane et al. [22] with 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid) supplemented with 10% calf serum were used. Overlay medium contained the medium of Yamane et al. with 0.7% agarose (Indubiose, Industrie Biologique Française, Clichy, France) and antibiotics. Phosphate buffered saline (PBS) of pH 7.2 was used to dilute mouse sera and inactivated EMC virus. PBS with 1% peptone (PBS-peptone) was used for infectious virus dilutions.

Haemagglutination

Haemagglutination reactions were done with sheep red blood cells as described by Martin et al. [13]. A virus suspension of 1.0 ml with a haemagglutination titre of 1:100 was defined to contain 100 haemagglutination units (HAU).

Determination of neutralizing antibodies

Virus neutralizing antibodies were determined with the plaque reduction test. Plaque titration and plaque reduction tests were done in plastic palettes (24-well tissue culture cluster dish; Catalog No. 3524; Costar, Cambridge, MA, U.S.A.). Dilutions were made in a 96-well palette (Catalog No 3596; Costar). Virus-containing fluids were titrated in 4-fold series by adding 0.05-ml quantities to 4 monolayers. After 40 min of adsorption at room temperature 0.3 ml aliquots of overlay medium were added. After 48 h incubation at 37°C plaques were developed with 0.05 ml of 0.02% neutral red. For plaque reduction tests, 0.025 ml serially diluted and decomplemented (30 min, 56°C) serum was mixed with 0.025 ml of virus and incubated at 37°C for 1 h. A 0.2-ml portion of PBS-peptone was added, and four 0.025-ml aliquots of each dilution were transferred to monolayers. Controls contained about 100 plaques/16-mm well. If a serum sample caused a 50% plaque reduction at a dilution of 1:10 it was described as containing an antibody concentration of 10 plaque neutralizing doses (PND_{50}). If no antibodies were detectable, the titre was quoted to be 0.1, which was the lowest possible level of detection.

Animals and immunization

Inbred male BALB/c mice were used at an age of 12 wk. Blood was obtained by retro-orbital punction. For intracutaneous (i.c.) immunization, mice were injected with a mixture of inactivated EMC virus and DDA (Eastman Kodak, Rochester, NY, U.S.A.) in 0.2-ml PBS at four divided sites (4×0.05 ml) in the neighbourhood of draining lymph nodes in axillae and groins. For intraperitoneal (i.p.) immunization mice were injected with viral antigen and adjuvant in a volume of 0.5 ml of PBS.

Peritoneal exudate cells

Immunized and normal mice were injected i.p. with 1.0 ml of thioglycollate broth. Two days later 2 ml of Eagle's medium was injected into the peritoneal cavity. After slight massage the animals were killed by cervical dislocation and the abdomen was opened. The peritoneal contents were harvested with a Pasteur pipette (1.2 to 1.8 ml). The peritoneal exudate cells were washed and counted. The cell suspensions were diluted to the required concentration, mixed with EMC virus and injected into the footpads of mice.

Assay for DH

DH was determined by measuring the increase in footpad thickness (footpad swelling test) 24 h after injecting an eliciting dose of diluted virus antigen in 0.05 ml PBS into the left hind footpad. The thickness of the footpad was measured with a semi-electronic footpad meter [23] with a sensitivity of 0.01 mm. Reactions were recorded against the day when the test dose of virus was injected, rather than the day upon which the reaction was measured.

Assay for local transfer of DH

Peritoneal exudate cells from thioglycollate-treated immunized and control mice were mixed with inactivated EMC virus. Numbers of 3×10^6 peritoneal exudate cells

and doses of 3000 HAU of EMC virus in volumes of 0.05 ml were injected in left hind footpads of mice.

Preparation of virus antigen

EMC virus was inoculated at a multiplicity of about 0.1 on monolayers of L cells in roller bottles and incubated for 18 h at 37°C. Culture medium without serum, containing virus and cell residues, was harvested and centrifuged at 1500 × *g*. Virus in the supernatant was concentrated 100-fold with the Amicon Corp., Danvers, MA, U.S.A., DIAFLO ultrafiltration system (HIP100, nominal molecular weight cut-off: 100 000). The concentrated virus suspension of 20 ml was treated with 50 mg trypsin (Difco Labs., Detroit, MI, U.S.A.) for 30 min at 37°C and passed over a Sephacryl superfine S-300 column (Pharmacia Fine Chemicals, Uppsala, Sweden) column (30 × 1 cm). Fractions with the highest haemagglutination (HA) titre were collected, pooled, dissolved in CsCl and ultracentrifuged for 16 h at 65 000 × *g* at 6°C in an SW 30 rotor of a Measuring & Scientific Equipment Ltd. (Manor Royal, Crawley, Sussex, England) Model 75 centrifuge. Ultracentrifugation in a CsCl gradient was repeated with fractions of the highest HA titre. The purified concentrated virus was passed over a Sephacryl superfine S-300 column. Fractions with the highest HA titre were pooled and diluted in PBS to a volume of 15 ml containing 6×10^5 HAU EMC virus per ml. The total amount was irradiated in a moving Petri dish (15-cm diameter) for 30 min with ultraviolet light (1600 erg/cm^2). No residual infectivity could be detected in 0.5 ml of the undiluted virus suspension while the haemagglutinating activity remained constant. The virus batch was divided in 0.2-ml portions and stored over fluid nitrogen.

In a number of experiments purified Semliki Forest virus was used as control antigen. Purified inactivated Semliki Forest virus was prepared according to the method described for EMC virus with a few modifications.

Statistical analysis

Results are expressed as the arithmetic mean of N independent observations ± SEM. Statistical significance was assessed by Student's t test.

Results

The influence of DDA and immunization route on the humoral antibody response to EMC virus

Groups of mice were immunized intracutaneously with various doses (500, 1 500 and 5 000 HAU) of inactivated EMC virus with or without added DDA (8 mg/kg). At days 5, 7 and 10 after immunization blood was collected from individual mice and neutralizing antibodies were determined in serum. Each mouse was bled only once. The lowest dose of virus (500 HAU) induced detectable antibody formation at day 7 in only one out of 20 mice (Table 1). Immunization with 3-fold (1 500 HAU) and 10-fold (5 000 HAU) higher doses of virus resulted in the appearance of neutralizing antibodies in a considerable proportion of mice. The antibody response was either not affected by DDA or slightly depressed.

TABLE 1

Effect of the adjuvant, DDA, on the development of neutralizing antibodies in mice immunized with inactivated Semliki Forest or EMC viruses by i.c. or i.p. injection[a]

Immunization schedule				Mean PND_{50} values (SE.; *N*) for neutralizing antibodies					
Antigen	Injection route	Antigen dose (HAU)	Adjuvant (8 mg/kg of DDA)	Day 5		Day 7		Day 10	
				Mean (SE; *N*)	*P* value[b]	Mean (SE; *N*)	*P* value	Mean (SE; *N*)	*P* value
EMC virus	i.c.	500	+	0.1 (0; 8)	n.s.	0.4 (0.3; 10)	n.s.	3.7 (0.8; 6)	n.s.
			−	0.1 (0; 8)		0.1 (0; 10)		1.7 (1.1; 6)	
		1 500	+	0.1 (0; 6)	n.s.	8.9 (7.1; 6)	n.s.	18 (10; 6)	n.s.
			−	0.1 (0; 6)		2.4 (1.0; 6)		112 (75; 6)	
		5 000	+	2.5 (0.8; 10)	n.s.	28 (7; 10)	<0.05	41 (27; 4)	n.s.
			−	5.8 (3.1; 10)		69 (20; 10)		74 (8; 4)	
EMC virus	i.p.	1 500	+	8.4 (8.3; 6)	n.s.	45 (13; 6)	n.s.	680 (350; 6)	n.s.
			−	0.1 (0; 6)		17 (9; 6)	<0.10	47 (2; 6)	<0.10
		5 000	+	5.8 (2.0; 14)	n.s.	77 (5; 8)	<0.025	500 (37; 8)	n.s.
			−	2.2 (0.9; 14)	<0.10	36 (17; 7)		350 (95; 8)	
Semliki Forest virus	i.p.	500	+	–		–		154 (38; 8)	<0.005
			−	–		–		12 (4; 8)	

[a] Compiled results of various experiments.

[b] Student's *t* test; n.s. = statistically not significant ($P > 0.05$).

In another set of experiments, groups of mice were immunized i.p. with 1 500 or 5 000 HAU of inactivated EMC virus, again with or without added DDA (8 mg/kg). At days 5, 7 and 10 after immunization, serum samples were taken from individual mice for determination of PND_{50} values (Table 1). A slightly but significantly increased mean PND_{50} was observed in mice immunized with DDA. A more pronounced enhancement of the humoral response by DDA was observed in mice immunized intraperitoneally with inactivated Semliki Forest virus.

Influence of DDA on the development of DH to EMC virus

To determine the effect of DDA on the induction of DH to EMC virus groups of mice were immunized intracutaneously with 500 HAU of EMC virus mixed with graded doses of the adjuvant (1 to 16 mg DDA/kg mouse). Control mice were injected with PBS or DDA alone. Six days after immunization mice were challenged by injection in the left hind footpad with 3 000 HAU of EMC virus (0.05 ml). Footpad thickness was measured after 3, 12, 24 and 48 h. DDA clearly caused an increase in footpad swelling to inactivated EMC virus when measured at 24 h (Table 2). Optimal responses (2-fold increase over control values) were obtained with a dose of 8 mg DDA/kg. Blood was collected from all immunized mice 24 h after challenge but none of the individual serum samples contained measurable neutralizing antibodies to EMC virus (results not given).

In order to determine the optimal interval between immunization and challenge,

TABLE 2

Effect of the adjuvant DDA on the induction of DH to EMC virus, as measured by the footpad swelling test

Immunization schedule[a]		Footpad swelling[b] measured at:			
Antigen	Dose of adjuvant (mg/kg of DDA)	3 h	12 h	24 h	48 h
None (PBS)	0	4.3	1.4	1.4 (0.4)[c]	0.5
	16	3.4	1.1	1.4 (0.3)	0.7
EMC virus	0	4.5	3.6	5.3 (0.6)	3.0
	1	5.9	4.8	6.0 (0.8)	3.6
	2	2.9	5.9	9.0 (1.2)	4.8
	4	4.0	6.1	10.4 (0.8)	5.3
	8	3.6	5.4	12.2 (0.3)	6.1
	16	3.2	4.8	8.5 (1.7)	3.9

[a] Groups of 5 mice were immunized i.c. with 500 HAU ultraviolet light-inactivated EMC virus mixed with graded amounts of DDA.

[b] All mice were challenged on day 6 by injection of 3000 HAU of virus in the footpad. Footpad swelling was measured at indicated time intervals and expressed in 0.1 mm.

[c] SE given in parentheses.

mice were immunized intracutaneously with 500 HAU inactivated EMC virus with or without addition of DDA (8 mg/kg). At various time points after immunization the mice were challenged with 3000 HAU of EMC antigen in the footpad. When the challenge was given as early as 3 days or as late as 10 days after immunization, footpad swelling was weak. With intermediate time intervals the responses were pronounced, with a maximum when the challenge was given on day 6 (Fig. 1). The antigen-specificity of the observed footpad swelling in mice immunized with EMC virus was analyzed using calf serum or inactivated Semliki Forest virus as controls in the challenge phase. In no case was footpad swelling observed (Table 3).

The following experiment was done to study the effect of antigen dose both on DH reaction and on appearance of neutralizing antibodies. Groups of 6 mice were immunized i.c. with graded amounts of EMC virus (5 to 50 000 HAU) with addition of DDA (8 mg/kg). After 6 days the mice were injected in the footpad with 3 000 HAU inactivated EMC virus. Footpad swelling was measured after 24 h and immediately thereafter blood was withdrawn from all mice for the determination of the neutralizing antibodies in individual sera. The results are shown in Fig. 2. The optimum dose of EMC virus for the induction of DH was found to be 500 HAU. With the relatively high doses of 5 000 and 50 000 HAU, footpad swelling was clearly less pronounced than with the lower doses (50 and 500 HAU) of antigen. The decrease of footpad swelling with higher immunization doses was concordant with the appearance of measurable neutralizing antibodies.

That the footpad reactivity was due to a cellular immune response was demonstrated by passive local transfer experiments. Donor mice were immunized i.c. with 500 HAU inactivated EMC virus with or without addition of DDA (8 mg/kg). Control mice received only PBS or DDA. Four days later all donor mice were injected i.p. with

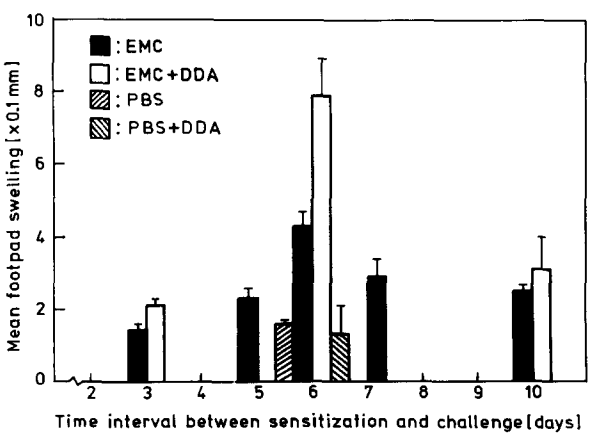

Fig. 1. Kinetics of development of DH to EMC virus as measured by the footpad swelling test. Groups of 6 mice were immunized i.c. with 500 HAU inactivated EMC virus, with or without addition of DDA (8 mg/kg). Control mice were included that received only PBS or DDA. At indicated time intervals after immunization, selected groups were injected in the left footpad with 3000 HAU of EMC virus, and footpad swelling was measured after 24 h. Vertical bars represent 1 SE.

TABLE 3

The antigen-specificity of the footpad swelling test in mice immunized with inactivated EMC virus

Immunization schedule[a]	Footpad swelling after challenge on day 6 with:					
	Diluted (1:100) calf serum		SFV (450 HAU)		EMC virus (3 000 HAU)	
	3 h	24 h	3 h	24 h	3 h	24 h
DDA	1.5	1.6 (0.5)[b]	2.4	0.6 (0.4)	4.3	0.9 (0.2)
EMC virus + DDA	0.9	0.2 (0.4)	3.4	1.0 (0.5)	4.4	10.1 (1.1)

[a] Groups of 5 mice were immunized i.c. with 500 HAU inactivated EMC virus and DDA (8 mg/kg mouse).

[b] Mean footpad swelling in 0.1 mm; SE given in parentheses.

1.0 ml thioglycollate broth. At day 6 thioglycollate-induced peritoneal exudate cells were harvested and prepared for local transfer. Aliquots of 3×10^6 cells were mixed with 3 000 HAU EMC virus and injected (0.05 ml) into the footpads of naive recipient mice. Footpad swelling was measured at 2, 6, 12, 24 and 48 h. As can be seen in Table 4 all mice developed footpad swelling immediately after the combined injection of antigen and cells. This swelling disappeared gradually, except in the mice which had received cells collected from donors immunized with both virus and DDA.

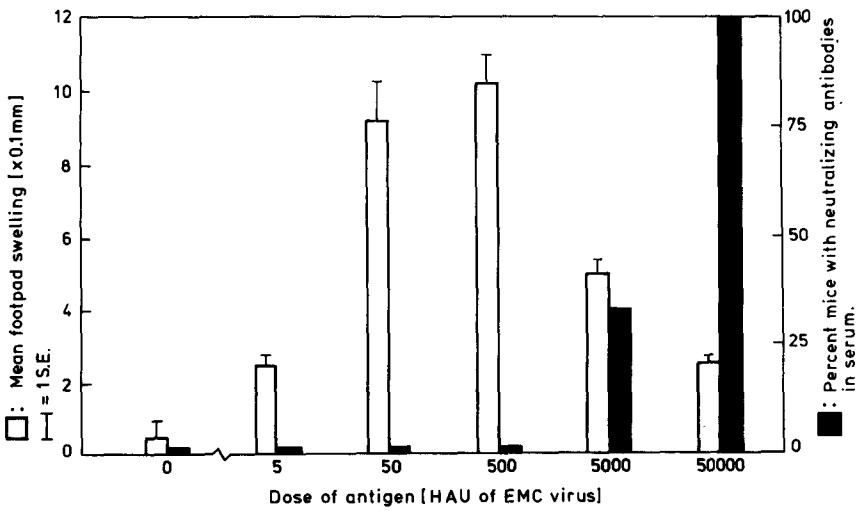

Fig. 2. Lack of relationship between DH reactivity and the presence of neutralizing antibodies in serum. Groups of 6 mice were immunized i.c. with graded amounts of inactivated EMC virus with addition of DDA (8 mg/kg). Control mice received only DDA. After 6 days all mice were injected in the footpad with 3 000 HAU of EMC virus and footpad swelling was measured after 24 h. On day 7 (24 h) post immunization the mice were also tested for the presence of neutralizing antibodies in serum.

TABLE 4

Passive local transfer of DH to EMC virus, as measured by the footpad swelling test

Donor mice[a]		Footpad swelling in recipient mice after local transfer at:				
Immunization antigen dose (HAU of EMC virus)	DDA (mg/kg)	2 h	6 h	12 h	24 h	48 h
0	0	10.0	8.3	6.3	5.4 (0.6)[b]	3.7 (0.9)
500	0	9.7	9.0	6.9	7.2 (0.8)	6.9 (0.9)
0	8	10.5	7.9	6.6	6.8 (0.7)	4.5 (0.8)
500	8	10.8	10.7	10.5	11.9 (0.8)	10.2 (1.2)

[a] Donor mice were immunized i.c. as indicated. Local transfer was performed at day 6: aliquots of 3×10^6 peritoneal exudate cells mixed with 3 000 HAU EMC virus (0.05 ml), were injected in the footpad of normal recipient mice (9 mice/group).

[b] Mean footpad swelling in 0.1 mm; SE given in parentheses.

DDA as an adjuvant for protective immunity

Groups of mice were immunized i.c. with graded doses of inactivated EMC virus (150, 500, 1 500 or 5 000 HAU) with or without addition of DDA (8 mg/kg). Control mice received only DDA or PBS. At day 6, separate groups of mice were tested for the presence of circulating antibodies, the presence of DH and resistance to infection with virulent EMC virus. The results are tabulated in Table 5. Specific antibodies were detected in sera of mice immunized with 1 500 or 5 000 HAU of EMC virus. In all groups DH was clearly enhanced by DDA (see also Fig. 2). To test protective immunity the mice were infected subcutaneously with 10 LD_{50} of virulent EMC virus and survival was recorded during 20 days. No mortality occurred later than day 13. Immunization provided protection against virulent EMC virus in a dose-dependent fashion and DDA enhanced this protection. DDA by itself had no protective effect against EMC virus infection. Protection afforded by immunization was slightly enhanced by DDA (statistically not significant).

Discussion

The lipophilic quarternary amine, DDA, enhanced the humoral response to inactivated EMC virus after i.p. immunization as well as the cellular immune response (DH) after i.c. immunization with the same antigen. DH to EMC virus, as measured by the footpad swelling test, was shown to be antigen-specific and could be transferred with peritoneal exudate cells to normal mice. The surface of EMC virus consists of nonglycosylated proteins arranged in a regular pattern of repeating subunits. Accordingly, the immunogenicity of the virus particle is partially thymus-independent, T-helper cells remaining necessary for the IgM/IgG switch [5]. The nucleocapsid proteins of EMC virus are interconnected by electrostatic and hydrophobic bonds

TABLE 5

Protective immunity, DH and antibody formation after immunization of mice with inactivated EMC virus with or without addition of the adjuvant DDA

Immunization schedule		Immunological parameters on day 6		Protection against virulent EMC virus infection[b] (no. survivors/no. infected)
Antigen (HAU) of EMC virus	Adjuvant (8 mg/kg of DDA)	No. of mice with neutralizing antibodies/ no. tested	DH as measured by the footpad swelling test[a]	
PBS	-	0/4	1.5 (0.6)	2/10
	+	0/4	2.2 (0.3)	1/10
150	-	0/4	5.9 (0.9)	4/10
	+	0/4	9.2 (1.2)	8/10
500	-	0/20	5.2 (1.1)	5/10
	+	1/20	9.5 (0.3)	5/10
1500	-	3/10	4.5 (0.4)	6/10
	+	5/10	8.4 (0.7)	7/10
5000	-	10/10	2.6 (0.8)	6/10
	+	8/10	8.4 (0.7)	10/10

[a] Groups of 5 mice injected with 3000 HAU inactivated EMC virus in the footpad; swelling measured after 24 h in 0.1 mm; SE given in parentheses.

[b] Subcutaneous infection with 10 LD_{50} EMC virus at day 6.

[10,16]. The regions responsible for these interactions could be involved in binding of DDA to the EMC virus particles. This, in turn could favour the induction of a cellular response [7, 21]. In previous studies [12, our unpubl. results] the use of DDA as an adjuvant was shown to cause a 5-fold increase in the DH reaction against Semliki Forest virus. In the present study, responses to EMC virus were increased only 2-fold by DDA. Presumably, attachment or insertion of DDA to double-layered lipid membrane antigens like sheep red blood cells [8,20] or Semliki Forest virus is more effective. The different immuno-enhancing effect of DDA on Semliki Forest and EMC viruses is also reflected in the humoral responses: the antibody response to Semliki Forest virus was considerably enhanced by DDA while that against EMC virus was only moderately affected.

DH to EMC virus, as measured by the footpad swelling test, was optimal at day 6. The most pronounced footpad reactivity was obtained with a smaller dose of immunizing antigen than that necessary to evoke detectable neutralizing antibodies. Higher doses of antigen did induce antibody formation but resulted in reduced footpad reactivity. A similar inverse relationship of DH and antibody formation has been found by other authors working with various antigens [11,18]. The association between the appearance of neutralizing antibodies and impairment of DH also provides further evidence for the virus-specificity of the footpad swelling after elicitation with inactivated EMC virus.

Immunization of mice by i.c. injection conveyed a state of resistance to infection with virulent EMC virus. When different doses of immunizing antigen were used, protection was not clearly correlated with the prevalence of neutralizing antibodies, but rather with the presence of DH.

DDA by itself did not affect resistance to EMC virus. In conjunction with vaccine it caused a suggestive (but statistically insignificant) increase in protection rate. Enhancement of DH by DDA suggests the presence of an increased number of specific T lymphocytes. This may increase resistance against infection, as these lymphocytes can specifically be stimulated by viral antigens to produce interferon-γ and macrophage activating factors [14]. However, B lymphocytes are primed after i.c. immunization with a single low dose of EMC virus, as can be concluded from the rather late appearance of neutralizing antibodies at day 10 (Table 1). This indicates that on day 6, besides specific T lymphocytes, committed B lymphocytes are present. Therefore, in our model, the relative involvement of both cell populations in protection against virulent EMC virus is not yet established.

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