Enhancement of Primary Antigen-Specific Resistance in Infant Mice with Divinyl Ether-Maleic Anhydride

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The early response of infant mice to foot-and-mouth disease viral antigens, measured by greater resistance and increased serum-neutralizing antibody, was enhanced by divinyl ether-maleic anhydride (DVE/MA). Preparations of known antigen concentration were used to study various parameters influencing this enhancement. Resistance and antibody levels were increased when DVE/MA was administered with aqueous or oil-emulsified antigens, whether given separately or mixed with the antigens, but was dependent on the dose and route of the DVE/MA and antigen. Enhanced resistance developed rapidly, was antigen-specific, and may have been related to the level of neutralizing antibody.

Divinyl ether-maleic anhydride (DVE/MA or pyran copolymer) is one of many polyanions that stimulate nonspecific antiviral activity in experimental animals. Interferon induction, antitumor activity, adjuvant activity, and altered reticuloendothelial system response have all been shown after DVE/MA administration (5, 14, 16, 17, 19), although the mode of action of the polymer is still unknown (1, 15).

In previous studies, a dose of 120 mg of DVE/MA per kg protected infant mice against the lethal effects of foot-and-mouth disease (FMD) virus (20), perhaps due to inhibition of virus multiplication *in vivo* (21). More recently, we reported (7) that 1.2 mg of DVE/MA per kg injected with oil-emulsified FMD vaccines prepared from inactivated tissue culture viral antigens enhanced mouse resistance to this virus. A broad range of DVE/MA concentrations failed to enhance demonstrable increases in early antibody formation to inactivated FMD vaccines in mice. Possible reasons for this may have included low levels of immunological competence, use of crude tissue culture antigen preparations, or inclusion of an oil adjuvant with the antigens (7).

In most studies of polyanion adjuvanting effects, sheep erythrocytes have been used as antigens; this system allows for detailed analysis of both the primary and secondary immune responses (3, 8, 11, 14, 15, 17). Data have been presented to indicate polyanionic stimulation of both B-cells (10) and T-cells (9). In studies reported here, concentrated or purified viral antigens, various injections procedures, and aqueous as well as oil-emulsified antigens were

used to evaluate the stimulating effect of DVE/MA on induced resistance and early antibody formation in a mouse model system. Experimental application of these findings in a host species naturally susceptible to FMD virus will be described in a following report.

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MATERIALS AND METHODS

Viruses and vaccines. FMD viruses, types C Resende (C-Res), O₁ Brugge (O₁Br), O₁ Caseros (O₁Cas), and A₂₄, were produced in primary bovine kidney or BHK-21 cell cultures. Infectious culture fluids were stored at -70°C and contained 10^{7.5} to 10^{8.0} mean lethal doses (LD₅₀)/ml for infant mice. Infectivity of the viruses used as antigens was inactivated with 0.05% acetylethyleneimine for 48 h at 37°C (13). Concentrated (18) C-Res, O₁Cas, and A₂₄ antigens and purified (23) O₁Br antigen were used in these studies. Aqueous antigens (AAg) were prepared by diluting inactivated virus to the appropriate concentrations with phosphate-buffered saline. Antigens in oil (OAg) were prepared by emulsifying inactivated virus in an equal volume of incomplete Freund adjuvant (13). For some experiments, selected concentrations of DVE/MA were incorporated in the AAg or OAg before use.

Divinyl ether-maleic anhydride. DVE/MA (lot no. XA124-77, Hercules, Inc., Wilmington, Del.) was prepared as described previously (20), and the pH was adjusted to 7.2 before use.

Mice. PIADC: M-1 (S) CV mice were used in these experiments (6). In one experiment in which the antibody response in mice of various ages was tested, 10 infant mice of the same age plus one mother mouse

were housed per cage. In another experiment, the antibody response was determined in 28-day-old male mice. In all other experiments, randomized 5- to 7-day old infant mice, housed 10 per cage with a nursing mother, were used.

Treatment and challenge procedures. Mice were injected with 0.05-ml amounts of DVE/MA and/or antigen intraperitoneally (i.p.), or subcutaneously in the neck (s.c._n) or in the inguinal regions (s.c._i). In initial experiments, mouse resistance was challenged with 100 LD₅₀ of virus (7). In all subsequent studies of induced antiviral resistance, groups of similarly treated mice were injected i.p. with serial 10-fold dilutions of virus (0.03 ml). Survival rates (protective index = $[\log_{10} \text{LD}_{50} \text{ control}] - [\log_{10} \text{LD}_{50} \text{ treated}]$) were computed 7 days later (20). Percentage computations were subjected to arc sine transformation before analysis, and all data were tested by analysis of variance (22).

Serum neutralization. Mice were bled by cardiac puncture under CO₂ anesthesia, and sera, pooled from 10 to 20 similarly treated mice per group, were heated at 56 C for 30 min and stored at -10 C. Antibody titers were determined in mice (12) and are reported as log₁₀ mean protective dose values. Sera unable to protect mice at a 1:2 dilution were considered antibody negative. To determine the specificity of the antibody produced, some sera were treated with 2-mercaptoethanol (13) before assay.

RESULTS

Development of antigenic competence. Various amounts of C-Res OAg were injected into mice that were 0, 1, 3, 4, 5, 7, 10, and 14 days old. Sera were collected 21 days postinjection (dpi) and tested for neutralizing antibody. Mice that were 3 days old (or older) when given at least 0.5 µg of OAg produced a measurable antibody response.

Influence of DVE/MA on antigen-induced resistance. Results of preliminary experiments showed that survival after i.p. treatment with 1.2 mg of DVE/MA per kg plus C-Res OAg (0.5 µg of antigen/mouse) was significantly ($P < 0.01\%$) greater than with either treatment alone. For up to 3 weeks after the double treatment, 50 to 90% survival against the lethal effects of 100 LD₅₀ of homologous virus was observed.

The enhancing effect of DVE/MA given with the viral antigen was not dependent upon the presence of the oil emulsion (Table 1). Analysis of variance indicated that there was no difference in the survival observed after i.p. injections of DVE/MA mixed with either C-Res OAg or C-Res AAg. Both treatments stimulated greater resistance than DVE/MA or either antigen preparation alone.

Because double treatments necessitated two injections (antigen s.c._n, polymer i.p. [7]), administration of a single injection of an antigen-DVE/MA mixture was assayed. Resistance was

not enhanced after s.c._n administration but was enhanced after s.c._i administration (Table 2). The greatest difference between either treatment alone and the double dosing was observed with 1.2 mg of DVE/MA per kg.

Comparing antibody levels and survival. In some experiments, the dose range of DVE/MA that would enhance the resistance stimulated by aqueous antigen and, possibly, stimulate antibody production was determined. Four days after s.c._i injections of O₁Br AAg (0.5 µg) plus various concentrations of DVE/MA or of DVE/MA alone, groups of mice were bled or injected with dilutions of homologous virus. Low doses of DVE/MA (0.24 to 2.4 mg/kg) were sufficient to enhance resistance, and 1.2 mg/kg was a sufficient dose to stimulate detectable antibody (Table 3).

Mice were injected s.c._i with various concentrations of A₂₄ AAg prepared in buffer or buffer containing DVE/MA (final polymer dose of 1.2 mg/kg) to estimate the minimal AAg dose required to elicit a measurable neutralizing antibody titer and to obtain an indication of survival rates relative to these titers. Some of the mice in each group were injected 4 days

TABLE 1. Survival response of infant mice treated with 0.5 µg of C-Res AAg or OAg, DVE/MA, or both and injected with C-Res virus at 2 dpi

Concn of DVE/MA (mg/kg)	DVE/MA alone	DVE/MA + OAg	DVE/MA + AAg
0.0	0.0 ^a	2.6	1.1
0.12	1.2	2.3	1.9
1.2	1.6	3.1	2.3
12.0	1.6	3.4	3.5
120.0	2.7	4.7	4.6

^a Survival rates = protective index values, where control titer was 10^{8.1} LD₅₀/ml.

TABLE 2. Survival of mice treated with DVE/MA, 0.05 µg of C-Res OAg, or both by different routes of administration^a

Concn of DVE/MA (mg/kg)	DVE/MA, s.c. _n		DVE/MA, i.p.		DVE/MA, s.c. _i	
	Con- trol	OAg, s.c. _n	Con- trol	OAg, s.c. _n	Con- trol	OAg, s.c. _i
0	2	15	3	30	5	50
0.12	0	20	0	25	0	54
1.2	5	23	12	65	8	78
12	3	13	78	85	65	90

^a Percentage of survival 7 days after 100 LD₅₀ of C-Res virus; mouse resistance challenged 4 days after treatment. Materials injected: s.c._n, subcutaneously in the neck; i.p., intraperitoneally; s.c._i, subcutaneously in the inguinal region.

later with dilutions of virus to determine their survival rates, and the other mice were bled for antibody assay (Table 4). Measurable antibody titers were not observed after treatment with less than 0.25 μg of AAg alone, although survival after administration of 0.125 μg of AAg per mouse was significant. When DVE/MA was mixed with at least 0.015 μg of AAg, however, resistance and antibody production were enhanced.

Kinetics of induced resistance and antibody release. Groups of mice were treated with 1.2 mg of DVE/MA per kg alone or in combination with either 0.5 μg of O_1Cas AAg or O_1Br AAg, or with either aqueous antigen alone. At daily intervals, mice from each group were bled or injected with dilutions of corresponding virus. Figure 1 presents the observed levels of resistance and serum-neutralizing antibody ti-

ters for the 7 days of each experiment. O_1Br AAg induced greater levels of resistance and higher antibody titers than O_1Cas AAg. The resistance mediated by either antigen treatment was enhanced by co-injection of DVE/MA, although the enhancement was more pronounced with O_1Cas AAg. In both instances, however, antibody was detectable 1 day earlier in the doubly treated mice than in mice that had been treated with either aqueous antigen alone.

Specificity of the enhanced resistance. Mice 28 days old were injected s.c. with 5 μg of AAg (C-Res , A_{24} , or O_1Cas) with or without 12 mg of DVE/MA per kg. Serum samples were collected 4 and 28 dpi for neutralization tests with each of the three virus types. The results showed that DVE/MA specifically increased the

TABLE 3. Survival rates and serum-neutralizing antibody titers of infant mice injected with 0.5 μg of O_1Br AAg and dilutions of DVE/MA

Concn of DVE/MA (mg/kg)	Survival ^a		Antibody titer ^b (DVE/MA + O_1Br AAg)
	DVE/MA	DVE/MA + O_1Br AAg	
0	0	2.7	0
0.24	0.4	3.6	0
1.2	1.2	4.1	2.29
2.4	1.2	3.7	3.36
12.0	1.4	4.2	2.50
24.0	1.4	4.2	2.57

^a Survival rates = protective index values, where control titer was $10^{5.2}$ LD₅₀/ml.
^b Antibody titer = log₁₀ mean protective dose in sera obtained 4 dpi.

TABLE 4. Survival rates and serum-neutralizing antibody titers of infant mice injected with 1.2 mg of DVE/MA per kg and dilutions of A_{24} AAg

Concn of A_{24} AAg ($\mu\text{g}/\text{mouse}$)	Survival ^a		Antibody titer ^b	
	A_{24} AAg	A_{24} AAg + DVE/MA	A_{24} AAg	A_{24} AAg + DVE/MA
0	0	0.8	0	0
0.015	0	1.8	0	2.29
0.031	0	1.5	0	2.64
0.063	0.1	2.4	0	2.43
0.125	0.9	2.2	0	2.64
0.25	1.6	3.1	2.29	2.50
0.5	2.2	2.6	2.43	2.71

^a Survival rates = protective index values, where control titer was $10^{7.6}$ LD₅₀/ml.
^b Antibody titers = log₁₀ mean protective dose in sera obtained 4 dpi.

FIG. 1. Kinetics of induced resistance and serum-neutralizing antibody release in infant mice injected with 1.2 mg of DVE/MA per kg alone or in combination with 0.5 μg of O_1Cas AAg (A) or 0.5 μg of O_1Br AAg (B) or with the antigens alone. Survival rate is calculated as protective index = $(\log_{10} \text{LD}_{50} \text{ control}) - (\log_{10} \text{LD}_{50} \text{ treated})$. Symbols: $\bullet$ — $\bullet$, survival after DVE/MA; $\bullet$ — $\bullet$, survival after AAg alone; $\bullet$ — $\bullet$, survival after DVE/MA plus antigen. Antibody levels are represented by the indicated bar graphs.

antibody titer only for the injected antigen. Slight cross-reactivity was observed with some of the sera collected 4 dpi. Treatment of these sera with 2-mercaptoethanol removed the neutralizing activity of the early samples but not of the sera collected 28 dpi.

DISCUSSION

This mouse model system for investigating antiviral enhancing effects of polyanions was developed because our concern has been related primarily to animal resistance against a specific virus (20, 21). The data presented here indicated that mice of the age used could produce serum-neutralizing antibody in response to injections of at least 0.5 μ g of OAg. It was furthermore indicated that the oil emulsion was not necessary to show increased resistance when DVE/MA was administered with the antigen (Table 1).

The possibility of an interferon-mediated resistance against FMD virus after DVE/MA administration has been considered minimal due to the transient nature and low titers of the interferon response (20). Mechanisms other than an interferon response have been suggested to interpret DVE/MA-mediated resistance (1, 14, 15). However, cells of the reticuloendothelial system clearly are involved (19). DVE/MA appears to express its biological activity when administered in the vicinity of peritoneal cells (Table 2). Part of this induced antiviral resistance may be related to inhibited virus release from the peritoneal cavity as suggested for another polycarboxylate, chlorite-oxidize oxyamylose (1). It should be noted that levels of DVE/MA too low to induce resistance when given alone stimulate both an antiviral state and increased antibody production when given with the antigen (Table 3).

The levels of resistance and serum-neutralizing antibody seen after administration of DVE/MA and 0.015 μ g of aqueous FMD antigen were comparable with those seen after administration of 0.25 μ g of AAg alone (Table 4). This comparability suggests that DVE/MA altered the host's ability to respond to very low antigenic stimulation. Braun has proposed (2-4) that the polyanionic nature of these compounds stimulates the function of primary antigen-handling cells; additional effects on the macrophages or other cells involved with the immune response may be due to cytotoxically released oligonucleotides.

Measured in terms of resistance and neutralizing antibody, mouse response to the O₁Br antigen was markedly better than the response

to the O₁Cas antigen (Fig. 1). This difference in antigenic responsiveness may also be reflected in the early (1 to 2 dpi) enhanced resistance observed with mice receiving DVE/MA plus O₁Br AAg. However, the eventual difference in resistance between O₁Cas-treated and O₁Cas-plus-DVE/MA-treated mice was greater than between the two O₁Br groups of mice. Enhancing resistance stimulated by otherwise relatively weak antigens could be an important consequence of including polyanions at the time of primary antigen exposure.

Neutralizing antibody was detected 1 day earlier after treatment with O₁Cas or O₁Br AAg plus DVE/MA than in mice that received either AAg alone. Increased resistance did not accompany the early, 2-dpi anti-O₁Cas antibody but did accompany 2-dpi anti-O₁Br antibody. This observation may reflect additional differences between the two antigens. Moreover, neutralizing antibody may not be an accurate parameter for measuring early polyanion-enhancing effects. Local antibody or cell-mediated immunity may be involved in these altered defense mechanisms, but their roles remain unknown.

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