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Protection of guinea pigs against local and systemic foot-and-mouth disease after administration of synthetic lipid amine (Avridine) liposomes

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Summary

Injection of the synthetic lipid amine, Avridine, in the form of liposomes, protected guinea pigs against the development of lesions from foot-and-mouth disease virus (FMDV) inoculated intradermally into the rear footpads. The animals were protected against the development of vesicles at the inoculation site as well as the systemic spread of virus. Maximal protection was obtained after intracardial injection of 5–10 mg doses of liposomal Avridine. Lower doses yielded decreased protection. Subcutaneous or intraperitoneal routes of liposomal Avridine injection were ineffective. Protection was maximal 0–24 h after injection of liposomes. Ethanol and emulsion formulations of Avridine could induce protection when injected intracardially but had toxic side effects. Guinea pigs protected against the first FMDV inoculation by liposomal and ethanol formulations of Avridine continued to be protected against lesions after a second inoculation 15–45 days later. FMDV protective antibody titers of these animals ranged from a low of $<1:10$ to $>1:1000$.

liposomes; Avridine; foot-and-mouth disease; CP20961; interferon

Introduction

In vitro serum neutralization tests and passive transfer of immune serum studies have demonstrated that foot-and-mouth disease virus (FMDV) antibody is protective both in vivo and in vitro [10]. The presence of other protective mechanisms is suggested by studies in which mice or guinea pigs showed varying degrees of protection against infection with FMDV after inoculation with interferon inducers or macrophage

activating agents [13–16]. These immunostimulants, or the immunologically active molecules that they induce, have potential use as adjuvants or antiviral prophylactic agents, and may also serve as tools for further exploring mechanisms of host protective immunity.

The synthetic lipid amine Avridine (*N,N*-dioctadecyl-*N'*,*N'*-bis[2-hydroxyethyl]-propane diamine), previously described as CP20961, has been shown to have several immunostimulatory properties. It is a potent adjuvant [1,12,17], an interferon inducer [6], a macrophage activating agent [12], and can enhance the bactericidal activity of neutrophils [18] and enhance lymphocyte cytotoxicity [12]. More recently it has been shown to reduce the severity of genital herpes in guinea pigs [20]. This compound, which has been demonstrated to be a powerful adjuvant for FMDV vaccine [7], was studied further to determine if it had potential as an antiviral agent for FMDV. Our results show that using the appropriate dose, route of inoculation and chemical formulation, Avridine treated guinea pigs are protected against the development of clinical lesions after a vigorous FMDV challenge.

Materials and Methods

Animals

Female Hartley strain guinea pigs were obtained from Dutchland Laboratories, (Dover, PA) and were used experimentally when they weighed between 300 and 325 g.

Virus

Foot-and-mouth disease virus type A_{12} strain 119 that had been adapted to guinea pigs was used throughout this study [8]. Infectious virus was obtained from fluid drained from vesicles which developed on the heelpads of the rear feet of guinea pigs 18 h after inoculation of the heelpads. Vesicular fluid was diluted with phosphate buffered saline (pH 7.4) (PBS) and stored in aliquots at -80°C. Virus infectivity was determined by inoculating intradermally 10 µl of a virus dilution in the right rear heelpad of each of 5 guinea pigs as described previously [9]. Virus infection was positive if a vesicular lesion developed at the inoculation site or any secondary site (tongue, other heelpads) within 7 days. Endpoints were calculated by the method of Spearman-Karber and are expressed as the virus dose that causes infection in 50% of the guinea pigs ($GPID_{50}$).

Interferon inducers

The ethanol formulation of Avridine was prepared as described elsewhere [7]. Briefly a 20 mg/ml solution was prepared by dissolving 140 mg of Avridine in 0.5 ml of warm (37°C) absolute ethanol, to which one drop of Tween 80 was added followed by 6.5 ml of PBS.

The emulsion formulation of Avridine was prepared as described previously [6] and contained 10 mg/ml Avridine, 10 mg/ml glycerine and 10 mg/ml polysorbate 80 in 0.01 M PBS, pH 7.0. The emulsion vehicle contained the same constituents except Avridine.

Liposomal Avridine was prepared by the ethanol injection method [2] and contained Avridine and dimyristoyl α-L-phosphatidylcholine (DMPC) in a 2 : 1 molar ratio. Briefly, Avridine (15 mg/ml) and DMPC (7.5 mg/ml) were dissolved in absolute ethanol and 1 volume of this solution was injected into 15 volumes of 0.15 M NaCl containing 0.005 M sodium phosphate buffer (pH 7.4). This preparation was then concentrated to 20 mg/ml using an XM100 ultrafiltration membrane (Amicon Corp., Lexington, MA).

The double-stranded polymer polyinosinic-polycytidilic acid (poly(I) : poly(C), sodium salt, Sigma, St. Louis, MO) was dissolved in PBS and used immediately.

The interferon inducers and PBS controls were injected in 0.5 ml volumes intracardially, intraperitoneally and subcutaneously into the nape of the neck.

Evaluation of protective immunity

Challenge of immunity by intradermal inoculations of virus into the tongue and right heelpad and the evaluation of vesicular lesions have been described in detail [9]. Unless otherwise indicated the protective immunity of guinea pigs in the current study was challenged by inoculating 10^3 GP ID_{50} of vesicular fluid virus intradermally into the right rear heelpad. The animals were examined every other day for 7 days for the development of vesicles at the virus inoculation site (primary site) and at secondary sites which included the tongue, the left rear heelpad, and the non-hairy surfaces of the front feet. An animal developing a vesicular lesion at only one secondary site is included with animals showing lesions at multiple sites in table headings.

Serum neutralization test

Serum neutralization of FMDV was assayed in mice by intraperitoneally inoculating 0.03 ml of serum–virus mixture containing 100 50% mouse lethal doses of FMDV into each of 10 suckling mice as described [8]. The neutralizing activity of each serum was calculated by the method of Spearman-Karber and expressed as $-\log_{10}$ of the 50% protective dose (PD_{50}).

Results

Dose of liposomal Avridine

Initial experiments were aimed at determining the dose of Avridine formulated in liposomes that might protect guinea pigs against FMDV. 1 h after intracardial injection of 0.1–10 mg of liposomal Avridine, the animals were inoculated with 10^3 $GPID_{50}$ of FMDV in the right rear heelpad. They were observed for up to 7 days for the development of vesicles at the virus inoculation site (primary site) and on the heelpad surfaces of the other three feet and the tongue (secondary sites).

Table 1 shows that control animals that had received 0.5 ml of PBS intracardially developed vesicles at the primary site and secondary sites after viral inoculations. Primary vesicles usually develop within 1–2 days of inoculation and secondary vesicles within 2–4 days. A 10 mg dose of liposomal Avridine protected 4 of 5 guinea pigs from developing primary vesicles and all 5 guinea pigs from developing secondary vesicles.

TABLE 1

Protective effects of different doses of intracardially inoculated liposomal Avridine administered 1 h prior to FMDV heelpad inoculation

Liposome dose (mg Avridine)	Animals[a] protected against vesicular lesions	
	Primary site[b]	Secondary site[c]
10	4	5
5	2	3
1	0	2
0.1	0	0
PBS	0	0

[a] Five animals per group.
[b] Each animal was inoculated in the right rear heelpad with 10^3 $GPID_{50}$ of FMDV one hour after liposome injection.
[c] The tongue and the footpads of the three uninoculated feet.

A lower level of protection was found with a 5 mg dose, even lower protection with 1 mg, and no protection with 0.1 mg. Similar dose–response results were obtained with another group of guinea pigs using the same experimental protocol but using the tongue as the primary inoculation site rather than the right heelpad (data not shown).

Duration of protective response

The duration of the protective effect induced by liposomal Avridine was studied next. Guinea pigs were injected intracardially with 10 mg of liposomal Avridine and 1, 24 and 72 h later they were inoculated in the right rear heelpad with FMDV. Table 2

TABLE 2

The effect of interval between intracardial injection of liposomal Avridine and FMDV footpad inoculation on protection

Interval[b] (h)	Animals[a] protected against vesicular lesions	
	Primary site[c]	Secondary site[d]
72	0	2
24	4	4
1	4	5
PBS control[e]	0	0

[a] Five animals per group.
[b] 10 mg of liposomal Avridine intracardially inoculated 72, 24 and 1 h prior to FMDV footpad inoculation.
[c] Each animal was inoculated in the right rear heelpad with 10^3 $GPID_{50}$ of FMDV.
[d] The tongue and the footpads of the three uninoculated feet.
[e] Control animals received 0.5 ml PBS injected intracardially 1 h prior to FMDV inoculation. Control and Avridine-treated animals were inoculated with FMDV on the same day.

shows that guinea pigs were protected against FMDV if the interval between Avridine injection and FMDV inoculation ranged from 1 to 24 h. Animals that were inoculated with FMDV 72 h after administration of liposomes had considerably less protection.

Route of liposome inoculation

To determine if the route of injection might influence the protective effects of liposomal Avridine, guinea pigs were injected with 10 mg of liposomal Avridine intracardially, subcutaneously in the nape of the neck and intraperitoneally. One hour later they were inoculated with FMDV in the right rear heelpad. Table 3 shows that animals intracardially injected with liposomes were protected against FMDV, but those that were injected by subcutaneous and intraperitoneal routes had little or no protection.

Comparison of Avridine formulations

Protection induced by other non-liposomal formulations of Avridine was compared with that induced by liposomal Avridine. Table 4 shows that intracardial injection of 5–10 mg of liposomal Avridine induced a high level of protection against FMDV infection. After injection of liposomes these and other animals in this study displayed no adverse effects. During the subsequent 2-month period they appeared healthy and to gain weight as well as normal animals of the same age. Intracardial injection of 5.0 mg of Avridine in the form of an emulsion produced a lower level of protection, and an even lower level of protection was produced by emulsion vehicle. After injection several animals treated with the Avridine emulsion were unsteady for 15–20 min. With the exception of the one animal that died, however, they were fully recovered the following day. Subcutaneous injection of 10 mg of the ethanol formulation of Avridine was not toxic and did not protect the guinea pigs, whereas 10 mg of this preparation injected intracardially was toxic for the guinea pigs. After injection the animals were unsteady and had difficulty breathing. Three of the animals died within 24 h and their

TABLE 3

Protective effects of liposomal Avridine inoculated intracardially, subcutaneously or intraperitoneally just prior to FMDV heelpad challenge

Route[b]	Animals[a] protected against vesicular lesions	
	Primary site[c]	Secondary site[d]
Intracardial	5	5
Subcutaneous	0	0
Intraperitoneal	1	1
PBS control	0	0

[a] Five animals per group.
[b] 10 mg of liposomal Avridine inoculated by each route.
[c] Right rear footpad.
[d] The tongue and the footpads of the three remaining uninoculated feet.

TABLE 4

Protective effects of different formulations of Avridine inoculated by different routes just prior to FMDV footpad inoculations

Avridine formulation	Route[b]	Toxicity[c]	Animals[a] protected against vesicular lesions	
			Primary site[d]	Secondary site[e]
Liposomes				
10 mg	i.c.	5/5	4/5	5/5
5 mg	i.c.	5/5	3/5	4/5
Emulsion				
5 mg	i.c.	5/6	2/5	3/5
vehicle	i.c.	5/5	1/5	1/5
Ethanol				
10 mg	s.c.	5/5	0/5	0/5
10 mg	i.c.	2/5	2/2	2/2
5 mg	i.c.	5/8	1/5	3/5
PBS control	i.c.	5/5	0/5	0/5

[a] Number of animals protected against FMDV/number of animals inoculated with FMDV.
[b] i.c. = intracardial; s.c. = subcutaneous.
[c] Number of surviving animals/number of animals injected with each formulation. Non-survivors died within 24 h of injection. The survivors were infected with FMDV as shown in the last two columns.
[d] Right rear footpad.
[e] The tongue and remaining three uninoculated feet.

lungs contained a diffuse yellow infiltrate that had the appearance and texture of insoluble Avridine. The two surviving animals recovered completely and were protected against FMDV infection. Intracardial injection of 5 mg amounts of the ethanol formulation induced similar toxic responses. Three of the five survivors were protected against virus spread to secondary sites.

Comparison of poly(I):poly(C) and Avridine

To determine if other interferon inducers could stimulate similar protective responses we compared the protective effects of Avridine liposomes to poly(I):poly(C). Table 5 shows that intracardial injection of 5.0 mg of poly(I):poly(C) protected 2 of 5 guinea pigs from developing primary vesicles and 4 of 5 guinea pigs from developing secondary vesicles. A lower dose of poly(I):poly(C) (1.0 mg) protected 4 of 5 guinea pigs against secondary vesicles. However, most protective activity was lost at 0.1 mg of poly(I):poly(C).

Avridine induced long-term immunity

Animals that were protected against FMDV inoculation after treatment with Avridine were studied further to determine if they developed long-term immunity. Table 6

TABLE 5

Comparison of the protective effects of liposomal Avridine and poly(I) : poly(C) intracardially injected just prior to FMDV footpad inoculation

Agent	Animals[a] protected against vesicular lesions	
	Primary site[b]	Secondary site[c]
Avridine liposomes (10 mg)	4	5
poly(I) : poly(C)		
5 mg	2	4
1 mg	0	4
0.1 mg	1	1
PBS control	0	0

[a] Five animals per group.
[b] Right rear heelpad.
[c] Tongue and the remaining three uninoculated feet.

TABLE 6

FMDV neutralizing antibody titers and protection against a second FMDV inoculation of Avridine-treated guinea pigs that were protected against the first FMDV inoculation

Animal No.[b]	Avridine formulation	Neutralizing antibody		Second FMDV inoculation[a]	
		Day serum obtained (DPI)	Titer (1/dilution)	Primary vesicles	Secondary vesicles
112	ethanol	14	6	-	-
113	ethanol	14	12	-	-
1	liposomal	44	120	-	-
2	liposomal	44	>1000	-	-
3	liposomal	44	>1000	-	-
4	liposomal	44	>1000	-	-
5[c]	liposomal	44	>1000	-	-
7	liposomal	44	316	-	-
8	liposomal	44	<10	+	+
9	liposomal	44	>1000	-	-
10	liposomal	44	<10	-	-
11	liposomal	44	40	+	-
14	liposomal	44	251	-	-

[a] Animals were inoculated intradermally with 10^3 $GPID_{50}$ FMDV in the left rear heelpad the day after serum samples were obtained as shown in column 3.
[b] Animals that were protected against FMDV inoculation in the right rear heelpad 1–24 h after intracardial injection of 10 mg of Avridine in ethanol or liposomal formulations.
[c] Developed a primary vesicle after first FMDV inoculation. All other guinea pigs in this column developed neither primary nor secondary vesicles after the first FMDV inoculation.

shows FMDV neutralizing antibody titers of animals that were protected against FMDV inoculation 1–24 h after intracardial administration of 10 mg amounts of Avridine in ethanol (animals 112, 113) or liposomal (animals 1–14) formulations. A wide range of antibody titers were found. Animals 112, 113, 8, 10 and 11 had titers of less than 1 : 100. The other animals had titers ranging from 1 : 120 (animal 1) to greater than 1 : 1000. Four control guinea pigs that had been injected intracardially with 0.5 ml PBS and then infected with FMDV were used in the same experiment as pigs 1–14 and had antibody titers of 1 : 2500 or greater at 44DPI (data not shown). Despite the wide range of antibody titers, all animals, with the exception of animal 8, were protected against a second FMDV inoculation in the left heelpad at 15 or 45 days after the first heelpad inoculation.

Discussion

In this study we have used a vigorous challenge inoculation with FMDV to test the protective effects of liposomal Avridine in guinea pigs. Virus is directly inoculated into heelpad tissue where the virus replicates and spreads via the blood to secondary sites; the tongue and the three uninoculated feet. Our results clearly show that intracardial injection of liposomal Avridine can protect guinea pigs against this vigorous FMDV challenge. For example, a total of 25 guinea pigs in five experiments (Tables 1–5) were intracardially injected with 10 mg of liposomal Avridine and shortly thereafter inoculated in the right heelpad with FMDV. Twenty-one of the 25 guinea pigs did not develop any lesions at all, and the four animals that developed a lesion at the inoculation site did not develop lesions at any secondary sites. In contrast, all of the 25 control animals that were intracardially injected with PBS developed lesions at the virus inoculation site as well as at secondary sites.

The guinea pig adapted virus used in this study is highly virulent for guinea pigs. As few as 10 pfu inoculated subcutaneously will cause systemic FMD [8] and a dose of 10^3 $GPID_{50}$, the challenge inoculation dose used in our studies, causes systemic disease in 100% of footpad inoculated animals. Furthermore, this dose is lethal for 10–30% of guinea pigs that weigh under 400 g. To protect guinea pigs against this challenge dose of virus requires 5 PD_{50} $\log_{10}$ per ml of passively transferred neutralizing antibody [10]. Therefore, Avridine must have induced a powerful immune response to protect against this virulent virus.

The results of several experiments suggest that there may be two mechanisms of protection operating in Avridine or poly(I) : poly(C) treated animals. One is the protective response generally seen with 10 mg of intracardially injected Avridine that prevents viral replication at the intradermal inoculation site. A second mechanism may be inferred from the several Avridine or poly(I) : poly(C) treated animals that developed vesicles at primary sites but not at secondary sites. Examples of these animals are found scattered throughout Tables 1–5. Because the primary sites of these animals are susceptible to exogenous virus, their secondary sites should also be susceptible to virus traveling via the blood from vesicles developing at the primary inoculation site. The development of primary but not secondary vesicles suggests that

these animals are demonstrating a second protective mechanism induced by Avridine or poly(I) : poly(C) which prevents virus spread from primary to secondary sites. The mechanism might be related to clearance of blood borne virus [11] after replication at the primary inoculation site by Avridine or poly(I) : poly(C) activated reticuloendothelial cells [3].

The protection against FMDV induced by liposomal Avridine was clearly dependent on the dose, route of injection and time of administration. In 300 g guinea pigs intracardially administered 5–10-mg doses of Avridine liposomes induced highly protective responses against FMDV that were effective for 0–24 h after liposome administration. Other routes of injection, lower doses, or longer intervals between the time of liposome administration and FMDV inoculation resulted in greatly decreased protection.

Avridine is hydrophobic and requires an organic solvent such as ethanol for solubilization. The addition of water-containing solutions to the ethanol solution leads to the formation of hydrophobic aggregates of Avridine. These aggregates were large and prominent in the ethanol/water solution of the 5 and 10 mg doses (10 and 20 mg Avridine per ml) that were intracardially injected. At 1.0 mg doses (2 mg per ml) the solution appears cloudy indicating the formation of much smaller aggregates. The diffuse yellow infiltrate in the lungs of the dead animals receiving 5–10 mg doses indicated that the large hydrophobic aggregates of Avridine were trapped in the lung and hence interfered with lung function. However, when Avridine was fashioned into liposomes with DMPC, the formation of large hydrophobic aggregates was averted, and the small liposomes, which are less than 2 μm in diameter (Hoffman, unpublished), can readily traverse the smallest blood vessels. Because all three Avridine formulations induced protection, it appears that toxic effects, but not protective effects, are dependent on formulation.

Injection of bacterial [5], viral and polynucleotide [19] interferon inducers stimulates peak levels of serum interferon within hours which decline during the following few hours or days. Avridine is a known interferon inducer [6] and FMDV an interferon-sensitive virus [15]. Thus, the 24 h period of antiviral protection induced by Avridine is probably related to interferon levels peaking a few hours after drug injection. Further support for an interferon mediated protective mechanism is suggested by the data of Table 5 in which poly(I) : poly(C) induced protection in guinea pigs against FMDV. Poly(I) : poly(C) is known to induce interferon after intracardial injection in guinea pigs [19].

Intracardial injection of Avridine was clearly superior to both intraperitoneal and subcutaneous routes. Although the intraperitoneal route is generally regarded as a suitable injection route for obtaining high vascular levels of test substances, injection of liposomes by this route induced only a low level of protection. This suggests that intraperitoneal liposomes, because of their size, surface charge or chemical composition are absorbed at rates too slow to provide the vascular levels necessary to stimulate protection. It appears that the intracardial route of injection is necessary so that large doses of Avridine traveling via the vascular system may quickly reach and activate the reticuloendothelial cells of the lung, spleen, liver and bone marrow. Activated cells may produce interferon [4] that inhibits viral replication and Avridine or interferon stimulated macrophages or other cells may develop other antiviral activities.

Several other effects of Avridine were observed in this study. Animals that were treated with Avridine and inoculated with FMDV, and which were subsequently protected against developing vesicles, developed a permanent state of immunity, i.e. they were protected against a second FMDV inoculation 15–45 days later. Many of the animals developed high antibody titers ($>1:1000$) which might suggest some viral replication occurred in these animals, but not enough to cause visible lesions. These high antibody titers can reasonably explain the protection of these animals to a second footpad inoculation several weeks later [9,10]. However, we found several animals that had low antibody titers ranging from $<1:10$ to $1:40$ but otherwise demonstrated complete or high levels of protection. These latter low antibody titers might protect some guinea pigs against systemic spread of FMDV but probably would not be able to confer protection at the footpad inoculation site [9,10]. High levels of protection in the presence of low antibody titers suggest that these animals were demonstrating a cell-mediated protective immune response to FMDV promoted by Avridine.

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