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Immunity to Foot-and-Mouth Disease Virus in Guinea Pigs: Clinical and Immune Responses

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Clinical and immune responses were determined for guinea pigs infected with different doses of foot-and-mouth disease virus (FMDV) type A_{12}, strain 119, administered by different routes. Vesicles developed on the tongue or heel pad 1 day after these areas were intradermally inoculated with FMDV. However, vesicles did not develop on the feet and tongue until 3 to 4 days after the intradermal inoculation of FMDV in the flank skin or after intracardiac or subcutaneous inoculation. Infected guinea pigs developed neutralizing antibody, immediate skin reactivity of the Arthus type (4 h), and delayed skin reactivity. In addition to a delayed skin response, the presence of a cell-mediated immune response to FMDV was shown by the specific production of macrophage migration inhibition factor by peritoneal exudate cells in response to FMDV. Kinetic studies showed that neutralizing antibodies were detected at 3 days postinfection, and Arthus and delayed skin reactivity were detected at 4 days postinfection. Some guinea pigs developed either mild or subclinical infections. Regardless of the dose of infectious virus, the route of inoculation, the severity of disease, or the time of clinical onset of disease, infected guinea pigs developed similar immune responses.

The humoral immune response to foot-and-mouth disease virus (FMDV) in cattle, swine, and other animals after infection or vaccination is characterized by the development of immunoglobulin M and immunoglobulin G antibodies which are capable of neutralizing infectious virus in vitro and in vivo (6, 19). In general, the level of circulating neutralizing antibody appears to be related to the degree of protective immunity shown by convalescent and vaccinated animals (7, 11; I. Nathans, Ph.D. thesis, Centraal Diergeneeskundig Institut, Amsterdam, 1965).

Cell-mediated immunity (CMI) has not been reported in foot-and-mouth disease, but in the studies just cited the presence of neutralizing antibodies may have obscured a protective role for CMI. The recent demonstration of CMI in mice infected with coxsackievirus B-3 (20), also a picornavirus, provides incentives for studying CMI to FMDV. The current study is part of a series aimed at determining the degree to which CMI and humoral immunity can contribute to the protection of various animal species against infection with FMDV.

Guinea pigs were chosen as experimental models with which to develop concepts and techniques to study CMI because of the similarities of clinical symptoms in these animals to those of swine and cattle. Unlike swine or cattle, guinea pigs cannot be infected via respiratory aerosols. However, inoculation with virus into the skin or tongue leads to clinical infection.

The objectives of the present study were to determine whether guinea pigs develop CMI after infection and to determine any differences in the clinical disease or resulting immune response of guinea pigs relative to the dose of administered virus or route of inoculation. The results show that infected guinea pigs develop subclinical, mild, and severe forms of foot-and-mouth disease, all of which lead to the apparent development of similar levels of CMI and humoral immunity.

MATERIALS AND METHODS

Virus. Guinea pig-adapted FMDV type A_{12} strain 119, provided by P. D. McKercher, had been passaged in guinea pigs 30 or 31 times by intradermally inoculating virus into heel pads and aspirating vesicular fluid 24 h later (6). The vesicular fluid had a titer of 10^7 plaque-forming units (PFU) when assayed in cultures of Mengeling-Vaughn Porcine Kidney (MVPK) cells (10). Concentrated, purified virus was obtained by polyethylene glycol precipitation of supernatant fluid from infected MVPK cells and ultracentrifugation on $CsCl_2$ gradients (18). Virus was inactivated with 0.1% binary ethyleneimine for 24 h at room temperature (4). The residual inactivator was then neutralized with 1% (wt/vol) sodium thiosulfate, fol-

lowed by extensive dialysis with phosphate-buffered saline, pH 7.2. Virus concentration was determined by spectrophotometry with the formula $E_{259\,nm}^{1\%} = 76$ (3). Inactivation was confirmed by the failure of 10, 1, and 0.1 μg of FMDV to infect guinea pigs after intralingual and heel pad inoculation or to cause lysis of MVPK cells. Purified inactivated virus was stored at 5°C.

Animal inoculations. Outbred, female Duncan-Hartley strain guinea pigs, weighing 300 to 400 g, obtained from this center's colony, were used for all experiments. Guinea pigs were infected with vesicular fluid diluted in phosphate-buffered saline to achieve the appropriate virus concentrations. Ten microliters of vesicular fluid was administered subcutaneously into the phalangeal pads of the right rear foot, intralingually into the anterior dorsal surface of the tongue (13), intradermally into the shaved flank, and intradermally by tracking virus into the metatarsal pad of the right rear foot (2). In this last method, two intradermal tracks approximately 1 to 2 cm long were made with a 26-gauge needle, and 5 μl of vesicular fluid was injected into each track as the needle was removed. Blood was inoculated by cardiac puncture with a 0.5-ml suspension of vesicular fluid diluted in phosphate-buffered saline containing 20 U of heparin per ml.

Guinea pigs were sensitized to *Mycobacterium tuberculosis* proteins by inoculation of 1 mg of killed *M. tuberculosis* H37RA (Difco Laboratories, Detroit, Mich.) emulsified in 0.5 ml of oil adjuvant consisting of nine parts Marcol 52 (Exxon Co., Bayonne, N.J.) and one part Arlacel A (Sandria Co., Bayside, N.Y.) (complete Freund adjuvant). Inoculations of 0.1 ml were given intradermally into each rear footpad, and inoculations of 0.3 ml were given intramuscularly in the neck.

Skin test. For skin tests, 100 μg of purified, inactivated virus in phosphate-buffered saline was injected intradermally into the shaved flank. Skin thickness was measured with skin calipers. Results are presented as specific increase in skin thickness, which represents the measurement (10^{-1} mm) of the skin fold at the test site minus the average thickness of the normal skin fold on both sides of the test area. Nonspecific increase in skin thickness at 4 h (1.7×10^{-1} mm) and 24 h (0.8×10^{-1} mm), induced by 100 μg of antigen in noninfected animals, was subtracted from the 4- and 24-h experimental skin test readings.

Serum neutralization test. FMDV neutralization tests were performed in suckling mice with a 50% protective dose (PD_{50}) assay (8). Fivefold dilutions of serum were mixed with equal volumes of a virus dilution such that the final mixture contained 100 50% mouse lethal doses per 0.03 ml. The serum-virus mixtures were incubated for 30 min at 37°C. Then 0.03 ml of each mixture was inoculated intraperitoneally into each of eight suckling mice. The numbers of mice surviving after 5 days were used to calculate the PD_{50}, which is the reciprocal of the $\log_{10}$ of the serum dilution protecting four of the eight suckling mice against 100 50% lethal doses of the virus.

MIF assay. A previously described macrophage migration inhibition factor (MIF) assay (1) was modified as follows. The medium was minimal essential medium with Earle salts supplemented with 25 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 5% fetal calf serum. Peritoneal exudate cells (PEC) were collected at 4 to 6 days after intraperitoneal injection of 25 ml of Marcol 52. The peritoneal cavity was opened with a mid-ventral incision and lavaged with 50 ml of minimal essential medium containing 5 U of heparin per ml. The PEC were washed twice with minimal essential medium, treated with 0.75% ammonium chloride (pH 7.2) to lyse residual erythrocytes, washed again, counted, and resuspended to 50×10^6 cells per ml. A 60-μl amount of cell suspension was dispensed with a Hamilton syringe (Pierce Chemical Co., Rockford, Ill.) into capillary tubes sealed at one end with Plasticene (Fisher Scientific Co., Pittsburgh, Pa.) and centrifuged at $500 \times g$ for 5 min. The capillary tubes were cut at the cell button-medium interface, and the short piece of capillary tubing containing the cell button was affixed to the inside of a migration inhibition chamber (Mini Lab Co., Ville de Laval, Quebec, Canada) with silicone grease. Each chamber contained two tubes and was sealed at the top with a coverslip and silicone. Medium (0.5 ml) containing 50 μg of purified protein derivative (Parke, Davis & Co., Detroit, Mich.) per ml was injected into the sealed chambers with a tuberculin syringe. The injection ports were sealed with silicone to prevent leakage. The chambers were incubated at 37°C in a water-saturated atmosphere containing 5% CO_2 for 24 h. The image of the capillary tube and the cell sheet that migrated from it was projected with a projecting microscope (Bausch & Lomb, Inc., Rochester, N.Y.) onto a sheet of paper, and the periphery of the area migrated by the cells was traced. The traced area was cut out and weighed. The percentage of migration inhibition (MI) was determined by the following formula: %MI = [1 − (average weight of area of migration with antigen/average weight of area of migration without antigen)] × 100. Each sample was assayed in quadruplicate. Results are expressed as mean values of groups of four or five guinea pigs. Standard errors for each test group were less than 20% and are not included in the data shown in the tables.

RESULTS

Effects of dose and route of infection on clinical and immune responses of guinea pigs. The highly infectious nature of the guinea pig-adapted FMDV type A_{12}, strain 119, is shown in Table 1; as few as 10 PFU of virus caused clinical disease in two of three guinea pigs infected subcutaneously with this virus. Higher doses of virus (10^3 and 10^5 PFU) caused clinical disease in all animals.

In subcutaneously infected animals, clinical disease was apparent after 3 to 4 days when vesicles developed on the four feet and the tongue. Vesicle formation was most consistent and apparent on the heel pads of the rear feet, where as much as 90 to 100% of the heel pad surface was covered by a vesicle or vesicles. Small vesicles (1 to 2 mm) appeared on the front feet which, in addition, often became erythe-

TABLE 1. *Clinical and immune responses of guinea pigs after infection with different doses of FMDV administered subcutaneously*

Guinea pig no.	Virus dose (PFU)	Vesicles[a]	Increase in skin thickness (mm × 10^{-1})[b]		Neutralizing antibody[b] (1/log PD_{50})
			4 h	24 h	
1	10^5	++	14	11	3.72
2	10^5	++	12	15	3.26
3	10^5	±	14	9	3.33
4	10^3	++	21	13	3.27
5	10^3	++	15	13	3.50
6	10^3	++	17	10	3.57
7	10^1	−	1	0	<0.30
8	10^1	++	18	12	3.33
9	10^1	++	12	8	3.20
10	10^0	−	0	2	<0.30
11	10^0	−	4	1	<0.30
12	10^0	−	3	1	<0.30

[a] Symbols: ++, vesicles on all four feet covering 50% or more of heel pad surface; +, vesicles on all four feet covering less than 50% of heel pad surface; ±, small vesicle on rear foot but no other lesions; −, no detectable lesions on any foot or tongue. Vesicles developed at 3 to 4 days p.i.

[b] Skin test reactivity and neutralizing antibody (PD_{50}) assayed at 25 days p.i.

matous, swollen, and scaly. In approximately 50 to 70% of the cases that showed vesicles on the heel pads, the tongue also developed vesicles. Healing of the heel pads, forefeet, and tongue was usually apparent 7 to 10 days after infection. Table 1 shows that all clinically infected animals showed severe signs of disease, except animal no. 3, which developed only a small vesicle on the heel pad of the foot used for infection. Despite this mild form of disease, this animal had an immune response of the same magnitude as the guinea pigs showing generalized lesions.

Neutralizing antibody titers and skin test reactivity of all guinea pigs were assayed at 25 days postinfection (p.i.) (Table 1). Clinically infected guinea pigs showed high levels of neutralizing antibody and skin reactions characterized by marked induration at both 4 and 24 h. Arthus (4 h) reactivity was usually 20 to 30% higher than the delayed (24 h) reactivity. Arthus reactivity was accompanied by erythema, which was first seen as a pink area 15 to 20 mm in diameter and which deepened in intensity to a red area 20 to 25 mm in diameter at 24 h (data not shown). The persistence of skin test reactivity at 24 h suggested a cell-mediated immune response to FMDV.

When guinea pigs were infected intracardially (data not shown) with the same viral doses shown in Table 1, a higher dose of virus (10^3 PFU) was necessary to initiate clinical infection, probably as a consequence of phagocytosis of some virus particles by the reticuloendothelial system before they could reach the appropriate target cells in which to replicate. At this dose of infectious virus, only one of three guinea pigs showed clinical disease. This clinically infected animal showed Arthus and delayed responses similar to the clinically infected animals shown in Table 1. However, one of the other two guinea pigs in this group which showed no signs of infection nevertheless showed an immune response comparable to that of the clinically infected guinea pig. Animals infected with 10^5 PFU of FMDV developed clinical disease, and their immune responses were comparable to those of the clinically infected guinea pigs shown in Table 1.

Specific production of MIF in response to FMDV. The production of MIF is an in vitro correlate of delayed-type hypersensitivity (9). Therefore, to further characterize the delayed skin response as being a result of a cell-mediated immune reaction, PEC collected from guinea pigs at 20 days p.i. were tested for their ability to produce MIF specifically in response to FMDV. In this experiment, we used a positive MIF control consisting of the PEC from guinea pigs immunized with killed mycobacteria in complete Freund adjuvant and stimulated with purified protein derivative. Table 2 shows that purified inactivated FMDV stimulated MIF production only by PEC from FMDV-infected animals; also, purified protein derivative stimulated MIF production only by PEC from mycobacteria-immunized animals. Thus, at 20 days p.i., PEC from infected guinea pigs could produce MIF, and the production of MIF was specific for

TABLE 2. *Specificity of MIF production in response to purified protein derivative and FMDV*

Animal treatment	% Migration inhibition	
	Purified protein derivative (10 μg/ml)	FMDV (50 μg/ml)
Control	−14	12
Mycobacteria immunized (complete Freund adjuvant)[a]	66	12
FMDV infected[b]	−5	52

[a] Animals were assayed for MIF 20 days after treatment with complete Freund adjuvant.

[b] Guinea pigs were inoculated with 10^4 PFU of FMDV subcutaneously and examined for MIF at 10 days p.i.

FMDV.

Kinetics of the immune response in FMDV-infected guinea pigs. The kinetics of both CMI and humoral immunity with virus neutralization assays, skin test, and MIF assays were examined. Figure 1 shows that neutralizing antibody was first detected on day 3 p.i., rose rapidly to a peak on day 6, and subsequently decreased slightly by days 10 and 20. Cowan and Trautman (6) showed that early antibody produced at 5 to 10 days p.i. was immunoglobulin M, which was replaced by immunoglobulin G antibody at 10 to 12 days p.i. In contrast to neutralizing antibody, Arthus reactivity, which primarily detects complement-fixing antibody of the immunoglobulin G_2 class (5), was first detected on day 4 and increased to maximum activity on days 10 and 20.

Delayed skin reactivity was also detected as early as day 4 p.i. and on days 4 to 6 was of the same magnitude as the Arthus reactivity. On days 10 and 20, delayed skin reactivity continued to increase. To further suggest a cell-mediated immune response to FMDV, substantial skin reactivity at 48 h could be detected on day 6 and continued to increase until day 20. The fact that MIF activity was detected on day 6 and persisted until day 20 further shows the early development and persistence of CMI to FMDV.

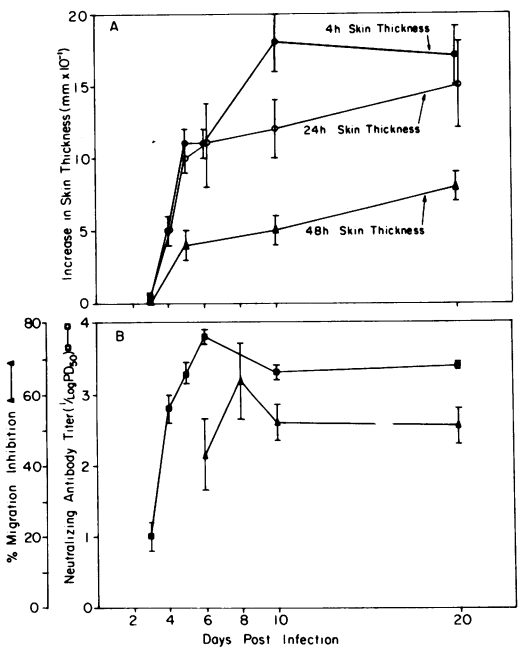

FIG. 1. *Time course of skin test reactivity (A) and production of MIF and neutralizing antibody (PD_{50}) (B) in guinea pigs inoculated subcutaneously with 10^4 PFU of FMDV type A_{12}, strain 119.*

From these data, the sequence of immune events appears to start with the initial production of immunoglobulin M on day 3 p.i., followed by low levels of immunoglobulin G production and the concomitant development of CMI on day 4.

Effect of different routes of infection on clinical and immune responses. Guinea pigs infected with 10^4 PFU of FMDV administered by different routes were observed for their clinical response to infection and then were skin-tested at 20 days p.i. (Table 3). All groups of animals showed strong Arthus reactivity and delayed skin reactivity. Despite the route of infection, no significant differences in the resulting CMI or humoral immunity could be determined. In similar experiments, PEC from animals infected by different routes produced significant amounts of MIF (50% or more migration inhibition) in response to stimulation with inactivated FMDV (data not shown).

Guinea pigs infected subcutaneously, intracardially, or intradermally in the flank skin developed vesicles at 3 to 4 days p.i., whereas intralingual- or heel pad-infected animals developed vesicles at the challenge site within 1 day p.i.; the disease generalized within the next 2 to 3 days. It was noteworthy that three of five animals infected via the flank skin in this experiment showed no signs of clinical disease, but nevertheless developed immune responses of the same magnitude as patently infected animals.

DISCUSSION

All infected guinea pigs that showed clinical disease had a strong humoral response as shown by virus neutralization assays and Arthus reactivity. The persistence of skin test reactivity at 24, 48, and 72 h strongly indicated CMI. CMI

TABLE 3. *Comparison of skin test reactivity of guinea pigs after infection by different routes*

Route of infection[a]	Clinical disease[b]	Increase in skin thickness (mm × 10^{-1})			
		4 h	24 h	48 h	72 h
Heel pad	5/5	22 ± 3[c]	13 ± 1	8 ± 1	5 ± 1
Flank skin	2/5	18 ± 4	12 ± 1	7 ± 1	3 ± 1
Subcutaneous	5/5	18 ± 2	12 ± 2	5 ± 1	2 ± 0.4
Intracardiac	5/5	20 ± 2	15 ± 2	8 ± 1	3 ± 1
Tongue	5/5	19 ± 1	14 ± 1	7 ± 1	4 ± 0.3
Heel pad and tongue	5/5	20 ± 1	15 ± 1	8 ± 1	6 ± 0.4

[a] Guinea pigs were infected with 10^4 PFU of FMDV and skin tested at 20 days p.i.

[b] Ratio of number of animals showing generalized vesicle formation on four feet to the total number of animals in each group.

[c] Mean ± standard error.

was confirmed by the demonstration that PEC from infected animals at various times after infection were stimulated to produce MIF in response to purified inactivated FMDV. In all of these studies purified binary ethyleneimine-inactivated FMDV was an excellent source of both skin test antigen and antigen used for in vitro stimulation.

Because all delayed skin responses seemed to be of similar magnitude regardless of the dose or route of infection, it was difficult to determine whether CMI in infected guinea pigs is equivalent to a strong, intermediate, or weak response. However, published reports (15, 16) on delayed skin reactivity to bovine serum albumin or to human serum albumin induced by inoculation of antigens in complete Freund adjuvant usually show that delayed responses increase from 24 to 48 h. In contrast, our results with FMDV-infected guinea pigs showed that delayed responses decreased after 24 h although they were still detectable at 72 h. Thus, in comparison with immunization with other antigens in complete Freund adjuvant, the CMI response to FMDV would have to be rated as weak or intermediate.

Infected guinea pigs showed subclinical, mild, and generalized forms of foot-and-mouth disease. A dose of 10^5 PFU of FMDV inoculated subcutaneously led to one example of mild disease, and a low dose of FMDV (10^3 PFU) administered by cardiac puncture led to one case of subclinical disease. The largest number of animals showing subclinical disease occurred in animals inoculated with 10^4 PFU in the flank skin (Table 3); three of five animals developed subclinical infections. Subclinically infected animals had CMI and humoral immunity of the same magnitude as clinically infected animals. Thus, the antigenic load necessary to induce this level of immunity in subclinically infected animals must be derived from replication of virus at nonvisible sites. Potential replication sites may be inferred from the work of Platt (17), who observed necrosis of the exocrine tissue of the pancreas, occasional necrosis of cardiac fibers, and epithelial lesions in the esophagus, prepuce, ear, and perineal regions of male and pregnant female infected guinea pigs. Subclinical infections with FMDV are not confined to guinea pigs because Henderson et al. (12) have reported inapparent infections in aerosol-infected cattle which resulted in a strong protective immunity.

Protective immunity in subclinically infected guinea pigs may also be related to the type of immunity induced by dose and route; low doses of antigen and intradermal inoculations preferentially stimulate CMI (14). A study of subclinical infections in guinea pigs may provide additional insights in mechanisms of host resistance to FMDV.

Graves et al. (11) have reported a high level of correlation between the FMDV neutralizing antibody titer induced by vaccination and the protection of cattle from infection. The current finding of a cell-mediated immune response to FMDV in guinea pigs suggests that CMI may also constitute part of the immune response to FMDV in swine and cattle. In addition, results in studies similar to this one might show that skin test sensitivity could be a useful tool in both laboratory and field studies of immunity to FMDV in swine and cattle.

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