The Effect of Parenteral and Oral Immunization on Encephalomyocarditis Infection in Mice[1] (34626)

T. G. LAWRENCE, JR. AND HUGH COLLINS
(Introduced by S. B. Formal)
Walter Reed Army Institute of Research, Washington, D. C. 20012

Encephalomyocarditis virus (EMC) is a member of the picornavirus group. Oral infection of mice produces viremia and fecal virus excretion which are followed by fatal encephalitis. EMC is similar to poliovirus physically and chemically, and Friedman and Maenza have suggested that EMC infection in mice be used as a model of poliovirus infection (1).

Oral attenuated virus vaccine prevents the subsequent infection of the bowel with a homotypic virulent challenge in both EMC (1) and poliovirus (2) infection. Parenteral inactivated vaccine does not convey firm protection against infection of the bowel with poliovirus (3), and antibody to poliovirus is found in duodenal secretory gamma A immunoglobulin after oral attenuated virus but not after parenteral inactivated virus (4).

Because of interest in the influence of the route of immunization on the subsequent resistance of the gut to infection, we have compared the effect of oral living-attenuated EMC with the effect of subcutaneous formalin-inactivated EMC on fecal excretion of virus following an oral virulent challenge.

Materials and Methods. In general, the procedures used by Friedman and Maenza were followed (1). Virus was grown in L-cell monolayers maintained in Eagle's basal medium containing 20% fetal bovine serum, and virus titers were determined by plaque formation (1). A virulent and an attenuated strain of the virus were used. Both were kindly provided by Dr. R. Friedman. An attenuated strain of EMC had been obtained by serial passage in L-cells (1). This strain maintained at —70°, produced asymptomatic viremia and fecal excretion when given orally to mice. The virulent strain of EMC was obtained by serial passage of virus in mouse brain. After a further 10 passages in brain by us, the infected brain homogenate was stored at —70° and was used to seed L-cell monolayers. The culture fluid containing either the virulent or the attentuated virus was harvested 48 hr after seeding and centrifuged. The clear supernatants were stored at —70°.

Animals. Fifteen- to 20-g female Balb/C mice were obtained from Dublin Animal Farms, Dublin, Virginia.

Preparation of formalin-inactivated virus. Culture fluid containing virulent virus was cleared of cell debris by centrifugation, and after passage through a 0.45-μ pore-size filter (Millipore) contained 1×10^8 PFU/ml. Formalin was added to a final concentration of 1:4000 and the resulting mixture was kept at pH 7.4, 37°, for 6 days. Inactivation appeared to follow first-order kinetics. Complete inactivation was confirmed by intracranial inoculation in weanling mice.

Immunization of mice. Mice referred to below as sc received 0.25 ml of formalin-inactivated culture fluid subcutaneously. This was given as an emulsion of equal amounts of Freund's complete adjuvant and virus suspension in a total volume of 0.5 ml. Ten days later the injection was repeated. Mice referred to as po were allowed to drink 1 ml of culture fluid containing approximately 10^8 PFU of living attenuated virus.

Challenge. Mice were deprived of food and water overnight, individually identified, and

[1] In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences–National Research Council.

then fed 1 ml of virulent culture fluid containing either 10^8 or 10^9 PFU. They were then housed in groups of five. Fecal specimens were collected as previously described (1). In some experiments small bowel virus content was estimated by washing the bowel lumen from jejunum to ileum with 2 ml of phosphate-buffered saline.

Antibody titers. Neutralizing antibody titers were determined by plaque reduction as described before (1), except that after 1 hr at 37° the reaction mixture was held overnight at 4°.

Bowel antibody. Sixty mice were divided into three equal groups of 20 each. One group served as an untreated control. One group was immunized with subcutaneous inactivated virus in Freund's complete adjuvant. Two injections, at days 0 and 10, were given. One group was fed living attenuated EMC on day 0. On day 17 the mice were sacrificed by ether anesthesia and the small bowels were rinsed with 2 ml phosphate-buffered saline containing 5% fetal bovine serum. Washouts from each group were pooled, centrifuged, and concentrated tenfold by negative-pressure dialysis. Neutralizing antibody titers were determined by plaque reduction. Serial twofold dilutions were made. An end point of 90% plaque reduction was used.

Passive immunization. Neutralizing antiserum to EMC was prepared in female New Zealand white rabbits. One ml of an emulsion of 10^8 PFU of EMC in Freund's complete adjuvant was distributed among the footpads. Rabbits were bled at 3 weeks. The neutralizing titer of the antiserum used was 1:10,000. The anti-EMC activity was restricted to the 7S region by sucrose-gradient ultracentrifugation. The antiserum was diluted in phosphate-buffered saline, and 1 ml of the appropriate dilution was injected subcutaneously into each mouse. Mice were challenged orally 24 hr later.

Labeling of rabbit IgG. Immunoelectrophoretically pure rabbit IgG was obtained by DEAE-cellulose (Selectacell, Brown Co.) chromatography in phosphate buffer, pH 8.0, 40 mmhos conductance. The protein was trace labeled with ^{125}I (New England Nuclear) by the method of McConahey and Dixon (5). The labeled IgG was then added to carrier rabbit serum, and 1 ml was injected subcutaneously. Mice were sacrificed 24 hr later. Blood and bowel washout radioactivity was determined in a well-type gamma spectrometer (Nuclear Chicago).

Results. Table I presents data concerning the fecal virus excretion and mortality in mice challenged 19, 30, and 60 days after immunization. The results are similar regardless of the time of challenge. Immunization with either oral living attenuated virus or parenteral formalin-inactivated virus protected against subsequent oral challenge with virulent virus. Fecal virus excretion was markedly suppressed in the survivors from both immunized groups. The few survivors in the control group also shed no virus. With one exception (Table I), there was no evidence of protection against paralytic disease in association with asymptomatic infection of the bowel.

Table II gives the results of virus recovery from bowel washouts. This group was challenged 3 months after immunization, and specimens were collected 2, 3, and 4 days after challenge. The results of this more sensitive search for virus confirm the previous data. In either orally or parenterally immunized mice, the virus content of the bowel lumen is markedly suppressed. The titer on the day 4 po group reflects a single animal which had a high concentration of virus.

Attempts to detect coproantibody in the feces of orally or parenterally immunized animals failed. However after tenfold concentration, the pooled bowel washouts from 20 orally immunized mice had demonstrable neutralizing activity. A dilution of 1:4 produced 90% plaque reduction. In contrast, no neutralizing activity was detected in bowel washouts from parenterally immunized animals. These studies were done in parallel with the 19-day challenge experiments shown in Table I. Parallel serum neutralizing antibody studies were also carried out. The geometric mean serum titer (log) of 13 orally immunized mice and of 15 parenterally immunized mice was 6.5 and 2.6, respectively (approximately an eightfold difference). Thus, the differences in bowel antibody

TABLE I. Mortality and Virus Excretion in Mice Challenged at 3 Weeks, 1 Month, or 2 Months After Oral Immunization with Attenuated Virus (po) or Parenteral Immunization with Formalin-Inactivated Virus (sc).

Challenge time (days)	Challenge dose	Group	Mortality	PFU excreted/fecal pellet		
				0–20	20–250	>250
19	10^9	C[g]	20/20	0[a]	5	15
	10^8	C	15/20	2	3	8
	10^9	sc	0/20	19	0	1[b]
	10^8	sc	0/20	20	0	0
	10^9	po	2/20	18	1[c]	1[c]
	10^8	po	2/20	19	1[c]	0
30	10^9	C	20/20	3	4	5
	10^8	C	18/20	3	2	4
	10^9	sc	4/20	16	2[d]	2[d]
	10^8	sc	4/20	8	2[d]	0
	10^9	po	2/20	16	2[c]	1[d]
	10^8	po	4/20	13	0	1[d]
60	10^9	C	28/30	3[f]	10	9
	10^9	sc	0/30	30	0	0
	10^9	po	5/25	20	2[d]	3[d]

[a] Number of mice excreting designated number of PFU/fecal pellet.
[b] No antibody (Ab) titer but survived.
[c] Died, no Ab titer.
[d] Died, Ab titer not done.
[e] One of the two died.
[f] Two of the three survived.
[g] Control.

recovery may be quantitative rather than qualitative and may merely reflect the differences in the level of the antibody response to the two methods of vaccination.

When ^{125}I-labeled rabbit IgG was given to mice subcutaneously, only 0.2% of the circulating radioactivity was recovered in bowel washouts. Nonetheless, mice were protected and fecal excretion of virus was prevented by the subcutaneous injection of 1 ml of a 1:300 dilution of rabbit antiserum (Fig. 1).

TABLE II. Virus in Washout of Small Bowel After Oral Challenge with Virulent Virus 3 Months After Immunization with Oral Attenuated Virus or Parenteral Formalin-Inactivated Virus.

	No. of washouts positive for virus			Geometric mean viral titer		
Day:	2	3	4	2	3	4
Control	9/9[a]	10/10	5/5	1070[b]	229,500	3220
sc	2/10	1/10	0/5	10	0	0
po	3/10	0/10	2/4	25	0	1145

[a] Number positive/number tested.
[b] Geometric mean number of PFU per 2 ml of small bowel washout.

Discussion. In poliovirus infection, oral immunization with living attenuated virus leads to secretory IgA antibody in the bowel lumen (4) and to solid protection against reinfection (2). Parenterally administered inactivated vaccine does neither (3, 4). EMC infection in mice resembles poliovirus infection in man in many respects (1), but the pattern of host resistance which attends immunization differs in that subcutaneous inactivated EMC virus prevents subsequent gut infection for at least 3 months after immunization. Neutralizing antibody was recovered from small bowel rinses after oral but not after parenteral immunization. Serum neutralizing antibody levels in the two groups were not comparable, however. The oral living-attenuated vaccine was more immunogen-

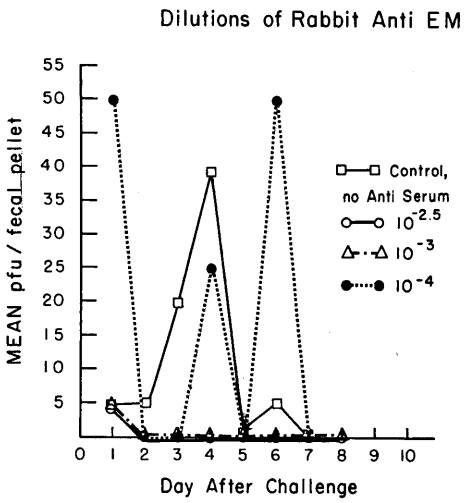

FIG. 1. Dilutions of rabbit anti-EMC. Daily pooled fecal excretion of virus following virulent oral challenge with virulent EMC at day 0. Each mouse received 1 ml of the indicated dilution of rabbit anti-EMC antiserum at day 1.

ic, reflecting perhaps the greater antigenic mass which resulted from infection and virus replication. It may be that the differences in bowel antibody content merely reflect this difference in immunogenicity and bear no relation to the route *per se*.

It seems probable, however, that the resistance to infection which is seen after parenteral immunization is unrelated to intestinal antibody. As noted, we found no coproantibody in this group. In addition, passive subcutaneous immunization with rabbit antiserum provided protection, in spite of evidence that less than 0.2% of the circulating IgG reached the lumen of the bowel.

EMC infection in mice differs from poliovirus in man in certain respects which may relate to the importance of local gut defenses. The human gut is very sensitive to poliovirus infection (6). The duration of fecal excretion may be prolonged, and inapparent infection, without nervous system involvement, is common. By contrast, a larger oral challenge is needed to produce EMC infection in mice. The period of fecal excretion is limited. Fecal excretion is almost invariably accompanied by viremia and followed by fatal encephalitis. Thus the gut, *per se*, may play a larger role in poliovirus infection than in EMC infection. There is no direct evidence that the fecal EMC virus recovered had replicated in the gut epithelium, and it is probable that such superficial epithelial cell infection is the type most susceptible to local antibody and least affected by circulating antibody (7). Evidence presently available indicates that both pancreas and gut lymphoid tissue are infected with EMC (1). Replication in these structures may be the major source of the virus found in the gut lumen. This possibility is supported by the fact that parenteral challenge with EMC is followed by the same pattern of fecal excretion that is seen after oral challenge (8). Circulating antibody may interfere with replication in these areas and thus account for the suppression of shedding that occurs.

Summary. Active immunization with parenteral formalin-inactivated EMC virus or with oral living-attenuated EMC virus protets mice against lethal oral challenge with virulent virus and prevents subsequent viral shedding in the feces. Similar results can be obtained by passively immunizing mice with rabbit anti-EMC antiserum. Coproantibody was recovered only from orally immunized mice, and was thus not correlated with resistance to fecal shedding of virus.

1. Friedman, R., and Maenza, R. M., J. Infec. Dis. **118**, 125 (1968).

2. Sabin, A. B., Michaels, R. H., Ziring, P., Krugman, S., and Warren, J., Pediatrics **31**, 641 (1963).

3 Sabin, A. B, *in* "Immunity and Virus Infection" (V. A. Najjar, ed.), pp. 211–232. Wiley, New York (1959).

4. Ogra, P. L., Karzon, D. T., Righthand, F., and MacGillivray, M., N. Engl. J. Med. **279**, 893 (1968).

5. McConahey, P. J., and Dixon, F. J., Int. Arch. Allergy Appl. Immunol. **29**, 185 (1966).

6. Sabin, A. B., Amer. J. Med. Sci. **230**, 1 (1955).

7. Smith, C. B., Purcell, R. H., Bellanti, J. A., and Chanock, R. M., N. Engl. J. Med. **275**, 1145 (1966).

8. Unpublished observations.

Received Oct. 23, 1969. P.S.E.B.M., 1970, Vol. 133.