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Enhanced Resistance Against Encephalomyocarditis Virus Infection in Mice, Induced by a Nonviable *Mycobacterium tuberculosis* Oil-Droplet Vaccine

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Female C57B1/10 mice injected intravenously (i.v.) with nonviable *Mycobacterium tuberculosis* Jamaica cells associated with oil-droplet emulsions (WCV) were highly resistant to the i.v. injection of encephalomyocarditis virus (EMCV). Resistance to infection (87% survival) was detected from 1 week to at least 12 weeks after injection of WCV. Mice vaccinated i.v. also were resistant to intraperitoneal, subcutaneous, or intramuscular virus challenge, but were not resistant to intracranial challenge. Mice vaccinated intraperitoneally also were resistant to virus infection, whereas WCV administered intramuscularly or subcutaneously did not protect mice from virus injected by any route. Less than 50% of WCV mice that survived virus challenge possessed serum anti-EMCV-neutralizing antibody (<1:10), and none had detectable (<1:10) serum interferon. Interferon was not detected in sera of WCV mice from 4 to 144 h after i.v. injection of EMCV. Studies concerning the effects of WCV on EMCV infection suggest that mice may be protected by mechanisms that inhibit early viral replication and spread of virus to the central nervous system.

It has been established in several systems that viable mycobacteria have the ability to stimulate host immunity against bacterial and viral diseases (1, 3, 4, 7, 9, 14, 24). In some instances, the resistance was more dramatic if animals were challenged with old tuberculin before injection of virus (1), and, in another instance, mycobacteria were not effective unless viral-specific immune serum also was administered (3).

Nonviable mycobacteria and various mycobacterial fractions also have been shown to enhance resistance to disease. It was reported by Remington and Merigan that mice injected with complete Freund adjuvant were more resistant to challenge with Mengo virus than mice given incomplete adjuvant lacking mycobacteria (21), and Gorhe showed that foot-and-mouth disease virus replication was inhibited in adult mice pretreated with Freund adjuvant (8). More recently, cord factor and its analogs were reported to elicit enhanced resistance to a *Salmonella* infection (25), and a material extracted from nonpathogenic mycobacteria conferred resistance to mice against Columbia SK virus challenge (13).

The present experiments show that nonviable mycobacteria cells prepared in an oil-in-saline emulsion will stimulate long-term resistance in mice to encephalomyocarditis virus (EMCV) infection. In addition, the results show that this resistance is not dependent upon systemic interferon stimulation and is not strictly a local phenomenon since virus and mycobacteria do not have to be injected into the same site.

MATERIALS AND METHODS

Tissue culture and media. Mouse L cells were obtained from R. K. Gerloff, Rocky Mountain Laboratory. The cells were maintained in Eagle minimum essential medium (Grand Island Biological Co., Grand Island, N.Y.; catalog no. F-15) supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin G (Eli Lilly and Co., Indianapolis, Ind.) per ml, and 1 μg of amphotericin B (E. R. Squibb and Sons, Inc., Princeton, N.J.) per ml. Dulbecco phosphate-buffered saline with Ca^{2+} and Mg^{2+} was used to wash monolayers.

Virus. EMCV was obtained from Michael Ross, National Institutes of Health, Bethesda, Md. A stock pool of EMCV, prepared from brains of 21-day-old mice 24 h after intracerebral infection, titered 6.5×10^7 plaque-forming units per ml on mouse L cells. Ten 50% lethal doses (LD_{50}) of virus was used to challenge mice unless stated otherwise. EMCV was titrated in a plaque assay on mouse L cells with a 0.75% methylcellulose overlay prepared in 2×-concentrated minimal essential medium (15).

Mice. Inbred adult female C57B1/10 mice reared at the Rocky Mountain Laboratory were used in all experiments.

Mycobacteria. *Mycobacterium tuberculosis* Jamaica was obtained from Carl Larson, University of Montana, Missoula. The culture was isolated from a fatal case of tuberculosis in 1933 by J. Freund and E. Opie (personal communication, George Kubica, Center for Disease Control, Atlanta, Ga.).

WCV. Whole-cell mycobacterial vaccines (WCV) were prepared as previously described (18). Briefly, 25.0 mg of autoclaved and heat-dried *M. tuberculosis* Jamaica was placed in a sterile tissue grinder equipped with a Teflon pestle (Scientific Glass Apparatus Co., Inc., Bloomfield, N.J.). Eight drops (approximately 320 μl) of a sterile light mineral oil (Drakeol 6VR, Pennsylvania Refining Co., Butler, Pa.) were added, and the mixture was ground to a smooth paste with the pestle attached to a drill press rotating at 800 rpm. The grinder containing the paste was heated in a 75°C water bath for 5 min, 10 ml of preheated (75°C) 0.15 M NaCl containing 0.2% Tween 80 was added to the tube, and the grinding was continued for another 5 min. The emulsion was heated at 65°C for 30 min and, in most instances, 0.2 ml containing 500 μg of *M. tuberculosis* was injected intravenously (i.v.). All emulsions were cultivated on Dubos plates and found to be uniformly negative for viable mycobacteria. A properly prepared emulsion microscopically showed *M. tuberculosis* associated with the oil droplets. A negative control emulsion of Tween-saline and oil (TSO) initially was prepared, as described above, without the *M. tuberculosis*. Any dilutions of the vaccines were made in 0.85% NaCl containing 0.2% Tween.

Interferon and neutralization assays. Assays for interferon were done on mouse L cells as described previously (16). Sera were titrated for neutralizing antibody against EMCV by plaque assay on mouse L cells.

RESULTS

Enhanced resistance to EMCV induced by varying concentrations of *M. tuberculosis* in WCV preparations. Mice inoculated i.v. with varying concentrations of WCV were challenged i.v. 4 weeks later with EMCV. The data (Table 1) show that 87% of the mice given either 500 or 250 μg of WCV survived virus challenge, whereas lesser concentrations of WCV were ineffective, as was a TSO emulsion prepared without *M. tuberculosis*.

Cell walls of *M. bovis* BCG, prepared in a Ribi press (22), also were used in oil-droplet vaccines to enhance resistance to EMCV. It was determined that 100 μg of i.v. injected cell wall vaccine protected mice against EMCV as well as did 500 μg of i.v. administered WCV (data not shown).

Effect of time after injection of WCV on resistance of mice to EMCV. The next experiment was done to study the kinetics of vaccine protection. The results (Fig. 1) show that highly significant protection of 87% was detected as early as 1 week after vaccination and persisted for at least 12 weeks. Other results (data not shown) indicated that mice injected i.v. with EMCV prior to, at the time of, or 1 or 3 days after vaccination were not protected. Furthermore, all control mice given the TSO emulsion died after challenge with a similar dose of EMCV. Because TSO never protected mice in this and other experiments, the emulsion was no longer prepared, and in succeeding experiments only normal unvaccinated mice were used as controls.

It also was determined that female WCV mice

TABLE 1. *Resistance against EMCV infection induced by varying concentrations of M. tuberculosis in WCV preparations*[a]

μg of *M. tuberculosis*/mouse	Survivors/total (%)	*P*[b]
500	7/8 (87)	<0.005
250	7/8 (87)	<0.005
100	2/8 (25)	NS
50	2/8 (25)	NS
10	1/7 (14)	NS
1	0/8 (0)	NS
None (TSO)	0/8 (0)	

[a] Mice were inoculated i.v. with 0.2 ml of WCV containing varying concentrations of killed *M. tuberculosis* Jamaica or with 0.2 ml of TSO emulsion prepared without *M. tuberculosis*. Four weeks after vaccination, mice were challenged i.v. with EMCV (10 LD_{50}). The experiment was terminated 30 days after virus injection.

[b] <0.005, Highly significant; NS, not significant (17).

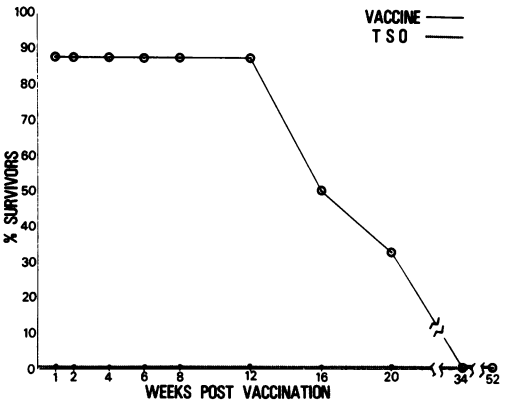

FIG. 1. *Effect of time after injection of WCV on resistance of mice to EMCV. Mice were inoculated i.v. with 0.2 ml of vaccine containing 500 μg of M. tuberculosis (WCV) or 0.2 ml of TSO emulsion prepared without M. tuberculosis. At various intervals thereafter, eight mice of each group were challenged i.v. with EMCV (10 LD_{50}). The percent survivors is highly significant for the first 12 weeks postvaccination ($P < 0.005$) (17).*

consistently were more resistant to EMCV than their age-related WCV male counterparts (data not shown).

Effect of i.v. administered WCV on resistance of mice to EMCV given by different routes. The previous experiment showed that mice given WCV i.v. were highly resistant to EMCV injected by the same route. Mice vaccinated i.v. were equally resistant to EMCV challenge by either the i.v., intraperitoneal (i.p.), intramuscular (i.m.), or subcutaneous (s.c.) route (Table 2). In marked contrast, 100% of the WCV mice died after intracranial injection with only 1 LD_{50} of EMCV. It also can be seen that, regardless of the route of virus injection, unvaccinated mice were equally susceptible to EMCV.

Effect of WCV administered by different routes on resistance of mice to EMCV. The above experiment has shown that i.v. vaccinated mice were equally resistant to EMCV injected by four different routes. The data (Table 3) indicate that, regardless of the route of challenge, i.p. vaccinated mice were as resistant to EMCV as were i.v. vaccinated mice. In contrast, i.m. and s.c. vaccinated mice were not protected.

Assays for serum anti-EMCV-neutralizing antibody and interferon in WCV mice that survived virus challenge. In a group of i.v. WCV mice that survived virus challenge, only 9% (4/47) of those challenged i.p. and 20% (10/49) of the i.m. challenged mice possessed circulating anti-EMCV-neutralizing antibody (>1:10); 50 and 51%, respectively, of the i.v. and s.c. challenged mice were positive (Table 4). Additional studies with WCV survivors killed 21 to 102 days after i.v. EMCV challenge showed similar results in that only 52% (44/84) possessed serum anti-EMCV-neutralizing antibody (data not shown).

It also was determined that none of the surviving WCV mice had detectable serum interferon and that neither WCV nor unvaccinated mice possessed serum interferon from 4 through 144 h after i.v. challenge with 10 LD_{50} of EMCV. Furthermore, interferon never was detected in sera of WCV mice at any of several different intervals after i.v. injection of the vaccine (data not shown).

Effect of WCV prepared with or without oil on enhanced resistance of mice to EMCV. Previous experiments by others (18, 22) have indicated that the effectiveness of mycobacteria cell wall vaccines in eliciting resistance to tuberculosis and enhancing tumor regression was enhanced by the presence of oil in the preparations. Thus, we prepared vaccines with and without oil to compare their effectiveness in eliciting enhanced resistance to EMCV. WCV prepared either way and injected i.v. afforded equal protection to mice from i.m. or s.c. EMCV challenge 3 weeks after vaccination (Table 5).

TABLE 2. *Effect of i.v. administered WCV on resistance of mice to EMCV given by different routes*[a]

Route of virus injection	Survivors/total (%)		
	WCV	Unvaccinated	P[b]
i.v.	44/54 (82)	0/15 (0)	<0.005
i.p.	47/54 (87)	1/15 (7)	<0.005
i.m.	49/54 (91)	1/15 (7)	<0.005
s.c.	47/54 (87)	1/15 (7)	<0.005
i.c.	0/15 (0)	0/15 (0)	

[a] Mice were inoculated i.v. with WCV containing 500 μg of *M. tuberculosis*. Two to nine weeks after vaccination, WCV and unvaccinated mice were challenged with EMCV. The i.v., i.p., and s.c. challenges consisted of 10 LD_{50} administered in 0.5 ml, the i.m. challenge consisted of 10 LD_{50} in 0.1 ml, and the intracranial (i.c.) challenge consisted of 1 LD_{50} in 0.03 ml. Thirty days after virus injection, the experiment was terminated.

[b] <0.005, Highly significant (17).

TABLE 3. *Effect of WCV administered by different routes on resistance of mice to EMCV*[a]

Route of vaccination	Survivors/total			
	i.v.[b]	i.p.	i.m.	s.c.
i.v.	5/6[c]	5/6	6/6	6/6
i.p.	5/6	6/6	6/6	6/6
i.m.	2/6	1/6	2/6	1/6
s.c.	0/6	0/6	1/6	0/6
None	0/6	0/6	0/6	0/6

[a] Mice were inoculated by one of four different routes with WCV containing 500 μg of *M. tuberculosis*. Four weeks later, age-related WCV and unvaccinated mice were inoculated with EMCV (10 LD_{50}) by different routes. Thirty days after injection of virus, the experiment was terminated.

[b] Route of EMCV injection.

[c] 6/6 = <0.005, Highly significant; 5/6 = <0.025, significant; 2/6, 1/6, and 0/6, not significant (17).

TABLE 4. *Serum anti-EMCV-neutralizing antibody in WCV mice that survived challenge with EMCV*[a]

Route of virus injection	Survivors with antibody/total survivors (%)
i.p.	4/47 (9)
i.m.	10/49 (21)
i.v.	22/44 (50)
s.c.	24/47 (51)

[a] Mice inoculated i.v. with WCV containing 500 μg of *M. tuberculosis* were subsequently inoculated with EMCV (10 LD_{50}). Thirty days later, the survivors were exsanguinated by severing the brachial vasculature, and sera were collected.

TABLE 5. *Effect of WCV prepared with or without oil on enhanced resistance of mice to EMCV*[a]

Vaccine and time after vaccination of virus challenge	Survivors/total (%)		
	i.p.[b]	i.m.	s.c.
3 weeks postvaccine			
+ Oil	10/12[c] (83)	10/12 (83)	11/12 (92)
− Oil	4/12 (33)	10/12 (83)	11/12 (92)
8 weeks postvaccine			
+ Oil	10/11 (91)	9/10 (90)	9/10 (90)
− Oil	2/10 (20)	3/10 (30)	5/10 (50)
Unvaccinated[d]	0/12 (0)	0/12 (0)	0/12 (0)

[a] Mice were inoculated i.v. with 500 μg of WCV prepared with or without oil. Three and eight weeks later, the mice were challenged with EMCV (10 LD_{50}) by one of three different routes.

[b] Route of EMCV injection.

[c] 10/12, 11/12, 10/11, and 9/10 = <0.005, Highly significant; 5/10 = <0.025, significant; 4/12, 3/10, and 2/10, not significant (17).

[d] Six unvaccinated age-related mice were challenged by each route at the two intervals.

Mice that had been administered vaccines prepared without oil, however, and injected with virus i.p. were 50% more susceptible than their counterparts that received WCV prepared with oil. Eight weeks after vaccination, WCV prepared in oil still protected mice (≥90%), whereas vaccines prepared without oil were 40 to 71% less protective depending on route of virus challenge.

Effect of i.v. administered WCV on viremia and spread of EMCV to various organs after i.m. or i.v. injection of virus. In a representative study concerning the effects of WCV on EMCV infection, 14 unvaccinated mice and 14 mice given WCV i.v. 4 weeks previously were injected i.m. with virus. At various intervals thereafter, three mice of each group were killed, and their sera and various organs were titrated for EMCV. Tissue weights of the two groups showed that livers and spleens of WCV mice were 55 and 241% heavier, respectively, than similar tissues from unvaccinated mice (Table 6). Five mice of each group were held for observation; all unvaccinated mice died by day 10 postinjection, whereas none of the WCV mice died by day 30. All three unvaccinated mice were viremic 48 h postinjection; three also had EMCV in the spleen, and two had virus in the liver (Fig. 2). Ninety-six hours postinjection, EMCV was detected in spleen and liver of all three unvaccinated mice, and two had virus in serum and brain. Substantial concentrations of virus also were present in the brain, spleen, and liver of one mouse that did not have a detectable viremia. In marked contrast to unvaccinated mice, EMCV was detected only twice in WCV mice, in the serum and spleen of the same mouse 72 h postinjection, and in both instances the virus concentration was extremely low.

In a second experiment, WCV and unvaccinated mice were injected i.v. with EMCV. Results were similar to those detected after i.m. challenge, except that viremia and the presence of virus in target organs were detected 24 h earlier in unvaccinated mice. EMCV was detected only

TABLE 6. *Average liver and spleen weights of WCV and unvaccinated mice*[a]

Group	Avg tissue wt (g) ± SEM[b]	
	Liver	Spleen
WCV	1.698 ± 0.102	0.370 ± 0.034
Unvaccinated	1.094 ± 0.064	0.109 ± 0.006

[a] Livers and spleens of nine age-related unvaccinated mice and nine mice that received WCV i.v. 4 weeks previously were weighed. By Student's *t* test, weight differences between similar tissues of the two groups were highly significant, $P = <0.001$.

[b] SEM, Standard error of the mean.

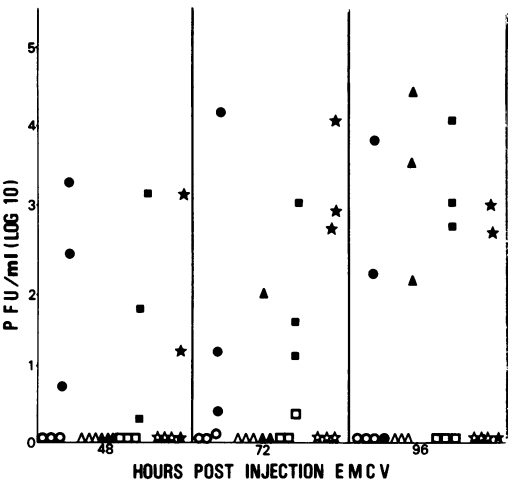

FIG. 2. *Effect of i.v. administered WCV on viremia and spread of EMCV to various organs after i.m. injection of virus. Age-related unvaccinated mice and mice given WCV i.v. 4 weeks previously were challenged i.m. with EMCV (10 LD_{50}). At each interval, three mice of each group were exsanguinated by severing the brachial vasculature, and sera were collected. The brain, spleen, and liver of each animal were removed, thoroughly rinsed in phosphate-buffered saline, and processed in minimal essential medium; the brain and spleen were emulsified as 10% suspensions, and the liver was emulsified as a 20% suspension. After freezing at −70°C, the sera and tissue supernatant fluids were titrated for EMCV. Symbols: WCV mice—○, serum; △, brain; □, spleen; ☆, liver. Symbols: Unvaccinated mice—●, serum; ▲, brain; ■, spleen; ★, liver.*

once in WCV mice: in the serum 72 h postinjection. Furthermore, all five unvaccinated mice held for observation died by day 9 postinjection, whereas only one of five WCV mice died by day 30.

DISCUSSION

The present experiments show that mice inoculated with WCV have an enhanced resistance against EMCV. This resistance is present as early as 1 week after the i.v. injection of vaccine, and highly significant host protection is maintained for at least 12 weeks. In addition, mice vaccinated either i.v. or i.p. are resistant to virus challenge by four different routes. The vaccine is most effective if it is prepared in oil, especially if virus challenge is by the i.p. route and/or occurs at an extended interval after vaccination. It has been assumed by others that the oil serves to present mycobacterial antigens more efficiently to cells and to provide a persistent source of antigenic stimulation (10).

The importance of systemic interferon in our system is doubtful. Interferon never was detected in sera of WCV mice that survived virus challenge or in an exhaustive search of sera of similar mice that were bled at several different intervals after injection of WCV, 10 LD_{50} of EMCV, or both WCV and EMCV. In other studies in which mycobacteria have been used to elicit resistance to virus infections, interferon either was not detected (23) or was not considered in host defense (1, 7, 14). In one instance, however, in which Freund complete adjuvant was shown to protect mice against foot-and-mouth disease virus, interferon was implicated as a mechanism in virus inhibition (8).

Other possible mechanisms by which WCV protects mice against EMCV may include enhanced ingestion and destruction of virus by macrophages. This may occur as a result of increased numbers of macrophages within the reticuloendothelial system, as shown by the significant hepatosplenomegaly (Table 6), or may be due to "activated" macrophages, since Kelly has shown that an *M. bovis* cell wall vaccine, prepared like our vaccine, activated guinea pig macrophages to produce an extracellular factor that kills *Listeria monocytogenes* (10). The importance of macrophages in our system will be studied in greater detail since preliminary data indicate that the i.p. injection of silica, which is toxic for macrophages and transiently depletes the macrophage population (2, 5, 19), completely abrogates resistance of WCV mice to i.m., i.p., or i.v. injected EMCV (data not shown). Thus, it may be that macrophages of WCV mice enhance resistance against EMCV by being more virucidal, releasing virucidal factors, increasing phagocytosis, or impeding spread of virus.

In other laboratories, enhanced immunity to virus infections has been elicited by nonviable extracts of *Brucella abortus* cells (6, 11, 26), heat-killed *Corynebacterium acnes* (20), and killed *C. parvum* (12). In one of these studies (11), interferon was induced by the extract, and enhancement of resistance to herpes simplex virus type 2 was compartmentalized; i.e., both immunopotentiator and virus challenge had to be confined to the peritoneal cavity. These results markedly differ from ours with EMCV. Furthermore, preliminary data indicate that compartmentalization of WCV is not necessary for protection against herpes simplex virus type 2, since i.p. vaccinated mice are protected against either an i.p. or i.v. virus challenge. Thus, it appears from this report and those of others that the mechanisms of enhancing resistance to virus infections are multiple and may depend on the immunopotentiator used and the virus infection being investigated.

The anti-viral resistance induced by WCV in our system does not appear to be mediated by an enhanced anti-viral antibody response (Table 4). Furthermore, preliminary results indicate that resistance cannot be transferred with sera, peritoneal exudate cells, or spleen cells of WCV mice to either lethally irradiated or nonirradiated normal recipients, and that neonatal thymectomy does not impair the ability of the vaccine to protect mice against EMCV (data not shown). Resistance also does not appear to be due to immunological cross-reactivity between EMCV and WCV, since the vaccine is effective against herpes simplex virus type 2. Thus, it appears that enhanced protection is a result of factors other than systemic interferon or conventional immune responses.

The uniform lethality of one LD_{50} of intracerebrally injected EMCV for WCV mice (Table 2) suggests that the vaccine protects mice against EMCV by the inhibition of early viral replication and subsequent spread of virus to the central nervous system. Preliminary data also suggest that the vaccine may be effective even after the early establishment of new foci of infection. Thus, it is our feeling that a new nonimmune mechanism of early virus resistance may be involved that evolves around the macrophage and may include release of nonspecific mediators, other than interferon, that can limit replication and spread of virus.

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