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Induction of Enhanced Resistance Against Encephalomyocarditis Virus Infection of Mice by Nonviable *Mycobacterium tuberculosis*: Mechanisms of Protection

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Nonviable *Mycobacterium tuberculosis* strain Jamaica suspended in oil-droplet emulsions was used to enhance resistance of mice against encephalomyocarditis virus (EMCV). The mycobacteria-injected mice were significantly resistant to 50,000 50% lethal doses of EMCV. Similar concentrations of virus in plasma of normal and mycobacteria-injected mice from 1 to 120 min after injection of EMCV showed that resistance was not a result of rapid elimination of virus from the circulation. Furthermore, survival of viremic mice indicated protective mechanisms were operative after EMCV had escaped primary surveillance. Resistance did not appear to be associated with the mouse major histocompatibility gene complex. The spleen was intimately associated with protection, and the thymus was nonessential for enhanced resistance to EMCV. Protection was significantly diminished by cyclophosphamide injected intraperitoneally from 3 days before to the day of virus challenge. Finally, silica given intraperitoneally 24 h before virus completely abrogated resistance of mycobacteria-injected mice to EMCV. These results suggest that macrophages functioning independently of T-lymphocytes are important effector cells in resistance to EMCV of mice injected with nonviable mycobacteria.

It is well known that mycobacteria, in particular the BCG strain, increase resistance to tumors (7, 11) and to bacterial (18, 28), viral (13, 21, 24), and parasitic infections (3-5). In a previous study, we showed that nonviable *Mycobacterium tuberculosis* strain Jamaica cells associated with oil-droplet emulsions stimulated long-lasting resistance of mice to encephalomyocarditis virus (EMCV) (12). This protection was shown to be systemic in that mice administered mycobacteria either intravenously (i.v.) or intraperitoneally (i.p.) were resistant to EMCV by four different routes of challenge. Furthermore, it was determined that resistance was not dependent on interferon. Pathogenesis studies suggested that mice might be protected by mechanisms that inhibited early viral replication and spread of virus to the central nervous system.

The present study was designed to determine the mechanism(s) of enhanced resistance induced by nonviable *M. tuberculosis* associated with oil-droplet emulsions by selectively removing or temporarily inactivating various components of the immune system. The results suggest that macrophages are important effector cells in this system and that they function independently of T-lymphocytes.

MATERIALS AND METHODS

EMCV, virus titrations, tissue culture and media, C57BL/10 mice, mycobacteria, and preparation of nonviable *M. tuberculosis* strain Jamaica oil-droplet emulsions have been reported in detail previously (12).

Mouse strains other than C57BL/10. Mice of strains other than C57BL/10 (see Table 4) were raised at the Rocky Mountain Laboratory and kindly provided by Bruce Chesebro.

Splenectomy. Spleens were removed under anesthesia after i.p. injection of 0.9 mg of sodium pentobarbital (Abbott Laboratories, North Chicago, Il.) per g of body weight for normal mice, and 1.2 mg of sodium pentobarbital per g of body weight for mice previously injected with nonviable *M. tuberculosis*. The incisions were closed with wound clips.

Thymectomy. Thymuses were removed by aspiration under constant vacuum pressure from precooled (4°C) neonatal mice <48 h of age. Incisions were closed with 3/8-inch (ca. 0.65-cm) circle taper needles attached to sterile, silicone-treated, silk-braided 6.0 thread (American Cyanamid Company, Pearl River, N.Y.).

Silica. Min-U-Sil no. 216 (Whittaker, Clark and Daniels, Inc., New York, N.Y.) was a gift from Carl Larson, University of Montana, Missoula. The Min-U-Sil was suspended in Dulbecco phosphate-buffered saline with Ca^{2+} and Mg^{2+} (12), sonically disrupted, and injected i.p. at a concentration of 200 mg/kg. Concentrations higher than 200 mg/kg were toxic for mice.

Cyclophosphamide. Cytoxan (Mead Johnson Laboratories, Evansville, Ind.) was suspended in Dulbecco phosphate-buffered saline and injected i.p. at a concentration of 300 mg/kg. Concentrations higher than 300 mg/kg were toxic for mice.

RESULTS

Effect of nonviable *M. tuberculosis* on resistance of mice to high concentrations of EMCV. Previous experiments showed that 10 50% lethal doses (LD_{50}) of EMCV killed 100% of normal mice but less than 20% of mice previously injected with nonviable *M. tuberculosis* suspended in an oil emulsion (12). The magnitude of this protection was particularly obvious with very high concentrations of virus; all mycobacteria-injected mice survived intramuscular challenge with 5,000 LD_{50} of EMCV, and statistically significant protection was detected after i.v. or intramuscular challenge with 50,000 LD_{50} of EMCV (Table 1).

Clearance of high concentrations of EMCV from blood of normal mice and mice injected with nonviable *M. tuberculosis*. A possible explanation for resistance of *M. tuberculosis*-injected mice to EMCV might involve the rapid removal of virus from the circulation. The data in Table 2 indicate this was not the case because virus titers in plasma of normal and mycobacteria-injected mice did not differ 1, 3, and 5 min after the i.v. injection of 10,000 LD_{50} of EMCV. Furthermore, additional experiments showed that virus titers in plasma were similar in normal and mycobacteria-injected animals 10, 60, and 120 min after the i.v. injection of similar amounts of virus (data not shown). A difference was detected, however, after sufficient time had elapsed for EMCV replication to occur; 24 h after injection of 10 LD_{50} of EMCV, concentrations of virus ranging from 10^2 to 10^4 plaque-forming units per ml of plasma were present in normal animals, and minimal, if any, virus was present in plasma of mice previously injected with *M. tuberculosis* (12).

Viremia and survival of mice injected with nonviable *M. tuberculosis*. We have observed and it recently has been reported that, once circulating virus is present in normal EMCV-injected mice, they uniformly die (23). We utilized this observation as a means to study enhanced resistance because survival of viremic mice would indicate that protective mechanisms were operative after virus had replicated. The data in Table 3 show that 10 of 20 mice injected i.v. with 100 LD_{50} of EMCV survived; 7 of these 10 survivors were viremic at 72 h, a time at which the initial virus inoculum would have been undetectable unless there had been viral replication (12). Additional evidence for viral replication was shown in the five survivors who

TABLE 1. *Effect of nonviable M. tuberculosis on resistance of mice to high concentrations of EMCV*[a]

LD_{50} of EMCV injected	Survivors/total (%)			
	i.v.[b]	*P* value[c]	i.m.[b]	*P* value
50	10/10 (100)	0.0000	10/10 (100)	0.0000
500	10/10 (100)	0.0000	10/10 (100)	0.0000
5,000	7/10 (70)	0.0015	10/10 (100)	0.0000
50,000	5/10 (50)	0.0163	5/10 (50)	0.0163
Control[d]	0/10 (0)		0/10 (0)	

[a] Mice inoculated i.v. with 500 μg of nonviable *M. tuberculosis* in oil-droplet emulsions were challenged 5 weeks later with different concentrations of EMCV.

[b] Route of EMCV injection. i.m., intramuscular.

[c] Treated versus untreated controls. According to Mainland (14) the levels of significance are <0.025 = significant and < 0.005 = highly significant.

[d] Normal mice were injected with the standard 10-LD_{50} challenge dose of EMCV.

TABLE 2. *Presence of EMCV in plasma of normal mice and mice injected with nonviable M. tuberculosis*[a]

Time (min) post-injection of EMCV	Plaque-forming units per 0.2 ml of plasma (log_{10}) in:	
	M. tuberculosis-injected mice	Normal mice
1	5.7, 5.7, 5.4 (5.6)[b]	5.7, 5.9, 6.0 (5.9)
3	5.9, 5.4, 5.4 (5.6)	5.7, 5.7, 6.0 (5.8)
5	5.4, 5.3, 5.7 (5.5)	5.4, 5.7, 5.6 (5.6)

[a] Three mice were injected i.v. 4 weeks previously with nonviable *M. tuberculosis* in oil-droplet emulsions, and three normal mice were injected i.v. with 10,000 LD_{50} of EMCV. At various intervals thereafter, blood was collected from the infraorbital sinus of each mouse, and the plasma was titrated for EMCV.

[b] Average titer.

TABLE 3. *Viremia and survival of mice injected with nonviable M. tuberculosis*[a]

Survivors (mouse no.)	Viremia at:	
	24 h	72 h
2	+	+
3	+	+
7	−	+
11	−	−
13	+	−
14	−	+
15	−	+
17	−	−
18	−	+
19	−	+

[a] Twenty mice inoculated i.v. with 500 μg of nonviable *M. tuberculosis* in oil-droplet emulsions were injected i.v. 4 weeks later with 100 LD_{50} of EMCV. At 24 and 72 h post-EMCV injection, 0.1 ml of blood was removed from the infraorbital plexus, and the plasma was checked for virus on ML cells. Virus in positive cultures was confirmed as EMCV by neutralization tests.

were not viremic 24 h after virus injection, but were viremic 48 h later (72 h postinjection). The decreased survival rate (50%) of mycobacteria-injected mice was probably due to the stress of the bleeding schedule.

Protection against EMCV in different mouse strains injected with nonviable *M. tuberculosis*. To determine whether resistance to EMCV was influenced by the mouse major histocompatibility gene complex, several strains of mice were injected with oil emulsions of nonviable *M. tuberculosis*. It was found that all mycobacteria-injected strains, regardless of *H-2* genotype, were resistant to EMCV (Table 4).

Effect of splenectomy before or after injection of nonviable *M. tuberculosis* on resistance of mice to EMCV. Mice injected with nonviable *M. tuberculosis* in oil-droplet suspensions have prominent hepatosplenomegaly (12). To determine the importance of the spleen in resistance to EMCV, spleens were removed from mice either before or after injection of the oil emulsion. Splenectomy performed before injection of *M. tuberculosis* had no effect on resistance of animals to EMCV (Table 5). In contrast, splenectomy performed after injection of *M. tuberculosis* markedly reduced resistance of mice to virus challenge. This reduced resistance was especially apparent if EMCV was administered i.v.; 20% of the mice survived, as compared with 85% of the mycobacteria-injected mice with spleens.

TABLE 4. *Protection against EMCV in different mouse strains injected with nonviable M. tuberculosis*[a]

Mouse strains	*H-2* genotype	Survivors/total (%)	
		M. tuberculosis injected	Normal
C57BL/10	*b/b*	7/8 (87)	2/8 (25)
BALB.B	*b/b*	6/8 (75)	0/8) (0)
(C57BL/10 × BALB.B)F_1	*b/b*	8/8 (100)	5/8 (62)
(C57BL/10 × A.BY)F_1	*b/b*	8/8 (100)	2/8 (25)
A.BY	*b/b*	4/8 (50)	0/8 (0)
(B10.D2 × BALB/c)F_1	*d/d*	7/8 (87)	2/8 (25)
(B10.A × A.BY)F_1	*b/a*	7/8 (87)	2/8 (25)
(B10.A × A)F_1	*a/a*	8/8 (100)	0/8 (0)

[a] Eight mice of each group were inoculated i.v. with 500 μg of nonviable *M. tuberculosis* in oil-droplet emulsions. Four weeks later the mice injected with *M. tuberculosis* and 8 normal mice of each group were challenged i.v. with 10 LD_{50} of EMCV. Thirty days after injection of virus the experiment was terminated.

TABLE 5. *Effect of splenectomy before or after injection of nonviable M. tuberculosis on resistance of mice to EMCV*[a]

Route of EMCV injection	Survivors/total (%)			
	Splenectomized before *M. tuberculosis* injection	Splenectomized after *M. tuberculosis* injection	Mice with spleens	
			M. tuberculosis	Normal
i.v.	18/20 (90)	4/20 (20)[b]	17/20 (85)	0/20 (0)
i.m.	19/20 (95)	13/20 (65)	18/20 (90)	0/20 (0)

[a] Mice were splenectomized either 1 week before or 3 weeks after the i.v. injection of 500 μg of nonviable *M. tuberculosis* in oil-droplet emulsions. Four weeks after injection of the *M. tuberculosis*, mice were challenged either i.v. or i.m. with 10 LD_{50} of EMCV. Sham splenectomy before or after injection of *M. tuberculosis* did not affect the resistance of mice to EMCV. Normal mice with or without spleens were equally susceptible to EMCV.

[b] Treated versus untreated injected with *M. tuberculosis*. $P = <0.005$ = highly significant (14).

Protection against EMCV in neonatally thymectomized mice injected with nonviable *M. tuberculosis*. Because T-lymphocytes are known to be important for protection against various virus infections, we questioned whether thymectomized mice injected with *M. tuberculosis* would be protected against EMCV. The data in Table 6 show that neonatal thymectomy did not limit the protective effect of *M. tuberculosis*; regardless of the route of virus challenge, 93% of the thymectomized mice survived. Macroscopic examination of survivors showed >80% without a thymus; histological examination of thymic areas from four mice without evidence of a thymus indicated no evidence of thymus remnants. Furthermore, in comparison with normal animals, low or absent hemolysin titers were detected in thymectomized mice 7 days after the i.p. injection of a 10% suspension of sheep erythrocytes. Preliminary data in athymic nude mice corroborate the thymectomy results in that nude mice also were protected against EMCV by nonviable *M. tuberculosis*.

Effect of silica on resistance to EMCV of mice injected with nonviable *M. tuberculosis*. The possibility that macrophages mediate *M. tuberculosis*-enhanced activity was investigated by the i.p. injection of silica, a known macrophage inactivator. The results in Table 7 show that silica completely abrogated the protective effect of *M. tuberculosis*. The total number of peritoneal exudate cells was similar in silica-treated and untreated mycobacteria-injected mice. However, differential cell counts of silica-treated mice indicated a ninefold decrease in macrophages and lymphoid cells and a twofold increase in neutrophils.

TABLE 6. *Protection against EMCV in neonatally thymectomized mice injected with nonviable M. tuberculosis*[a]

Group	Survivors/total (%)	
	i.p. injection	i.m. injection
M. tuberculosis + thymus	34/38 (90)	24/26 (93)
M. tuberculosis − thymus	26/28 (93)	26/28 (93)
Normal + thymus	0/10 (0)	0/10 (0)
Normal − thymus	1/10 (10)	0/10 (0)

[a] Neonatal thymectomies of precooled (4°C) mice <48 h old were done by aspiration. The treated animals were weaned at 30 days of age, fed autoclaved food, and maintained on water containing 10 mg of neomycin and 100,000 U of polymyxin B sulfate per liter. Approximately 8 weeks after weaning, mice were injected i.v. with 500 μg of nonviable *M. tuberculosis* in oil-droplet emulsions and challenged with 10 LD_{50} of EMCV 4 weeks later. Nonthymectomized littermates were held separately so that appropriate age-mated controls would be available for all experiments. All thymectomized survivors were macroscopically examined for thymus (>80% were free of thymus tissue), and thymic areas of four survivors without macroscopic evidence of a thymus were examined histologically by W. J. Hadlow for evidence of thymic remnants—none was detected.

TABLE 7. *Effect of silica on resistance to EMCV of mice injected with nonviable M. tuberculosis*[a]

Group	Survivors/total			
	i.p.	i.v.	i.m.	None
M. tuberculosis + silica	0/20	0/20	0/20	20/20
M. tuberculosis − silica	18/20	20/20	20/20	
Normal + silica	0/20	0/20	0/20	20/20
Normal − silica	0/20	0/20	0/20	

[a] Mice were injected i.v. with 500 μg of nonviable *M. tuberculosis* in oil-droplet emulsions. Four weeks later they were injected i.p. with 200 mg of silica and then challenged with 10 LD_{50} of EMCV 24 h later. i.m., intramuscular.

Effect of cyclophosphamide on resistance to EMCV of mice injected with nonviable *M. tuberculosis*. The effect of cyclophosphamide on resistance of *M. tuberculosis*-injected animals to EMCV was evaluated by varying the time at which the immunosuppressant was injected in relation to EMCV (Table 8). Cyclophosphamide markedly decreased, but did not completely abrogate resistance of mice if it was given from 3 days before to the day of EMCV injection. Administration of the drug 2 days before EMCV resulted in optimal abrogation of resistance (27% survivors); at this time there was a threefold decrease in the total number of peritoneal exudate cells after the i.p. injection of cyclophosphamide in treated as compared with untreated mycobacteria-injected mice. Differential cell counts, however, indicated no significant difference in the percentage of macrophages, lymphoid cells, and neutrophils.

TABLE 8. *Effect of cyclophosphamide on resistance to EMCV of mice injected with nonviable M. tuberculosis*[a]

Mouse group	Survivors/total (%)	P^b
Cytoxan injected		
−3 days[c]	20/40 (50)	0.0017
−2 days	11/40 (27)	0.0000
−1 day	19/40 (48)	0.0006
0	24/40 (60)	0.0098
+1 day	33/40 (82)	
+3 days	32/40 (80)	
Controls		
M. tuberculosis + cytoxan (no EMCV)	40/40 (100)	
M. tuberculosis + EMCV (no cytoxan)	38/40 (95)	
Normal mice + EMCV (no cytoxan)	1/40 (2.5)	

[a] Mice were inoculated i.p. with 500 μg of nonviable *M. tuberculosis* in oil-droplet emulsions. During week 5 post-*M. tuberculosis* injection, mice were inoculated i.p. with 300 mg of cyclophosphamide per kg and 10 LD_{50} of EMCV.

[b] Treated versus *M. tuberculosis* mice injected only with EMCV. According to Mainland (14) the levels of significance are <0.025 = significant, <0.005 = highly significant. Except as noted, all other probability values are >0.025.

[c] Time of cytoxan injection in relation to EMCV injection.

DISCUSSION

The present study was initiated to define the in vivo mechanism(s) responsible for enhanced resistance to EMCV of mice injected with nonviable *M. tuberculosis*. It was determined that mycobacteria-injected mice were significantly resistant to 50,000 LD_{50} of EMCV. Furthermore, similar concentrations of virus in plasma of normal and mycobacteria-injected mice from 1 to 120 min after i.v. injection of EMCV indicated that a possible mechanism of resistance was not due to rapid elimination of virus from the circulation. In corroboration of these results, it was shown that a protective mechanism was operative after EMCV had escaped primary surveillance; 7 of 10 mice that were viremic 72 h after virus injection survived until termination of their respective experiments. Supportive evidence that viremia had indeed developed in these mice was shown by the 5 of 10 survivors

who were not viremic 24 h after injection of EMCV, but were viremic 48 h later (72 h post-injection).

Several strains of mice with different *H-2* genotypes were protected equally well against EMCV by the oil emulsion preparation of nonviable *M. tuberculosis*. This result indicated that protection apparently was not associated with the mouse major histocompatibility gene complex. These results are in contrast to those of Civil and Mahmoud who showed that viable BCG induced nonspecific resistance to the multicellular helminth parasite *Schistosoma mansoni* in only certain strains of inbred mice (3); BALB/c, C3H/He, and CBA mice developed no or minimal nonspecific protection. Studies are in progress to determine whether nonresponder strains of mice to BCG similar to those used by Civil and Mahmoud, and other strains we have not previously tested, differ in their resistance to EMCV and other viruses after injection with oil emulsion preparations of nonviable *M. tuberculosis*.

Because of our previous detection of hepatosplenomegaly in mycobacteria-injected mice (12), the importance of the spleen in enhanced resistance to EMCV was evaluated. Interestingly, if the spleen was removed after injection of mycobacteria, resistance of mice to EMCV was markedly decreased, indicating that resistance was intimately associated with splenic function. If the spleen was removed before injection of mycobacteria, however, antiviral resistance was not affected. It would appear, therefore, that absence of the spleen led to an alteration in the normal distribution of *M. tuberculosis* after i.v. injection which resulted in localization of mycobacteria in areas that were entirely sufficient to enhance resistance to a lethal challenge of EMCV. The spleen, therefore, was not the only organ which harbored effector cells in this system.

The thymus was shown to be nonessential in establishment of enhanced antiviral resistance by nonviable *M. tuberculosis*, as it has previously been shown to be unimportant in thymectomized mice injected with the immunopotentiator pyran and then challenged with herpes simplex virus type 2 (17). In our studies, neonatal mice thymectomized at <48 h of age were as resistant to EMCV as their littermates that possessed a thymus or that had been sham thymectomized. Supportive evidence that the T-lymphocyte was not important was shown by our preliminary data in which resistance to EMCV was established in athymic nude mice injected with nonviable *M. tuberculosis*-oil-droplet emulsions. Thus, at least one mechanism of resistance to EMCV after injection of mycobacteria appears to involve a component of the immunological system other than the T-lymphocyte.

The most likely candidate appears to be the macrophage. The importance of the macrophage in our studies was shown by the complete abrogation of resistance to EMCV after the i.p. injection of silica, which is known to be toxic for macrophages (1, 6, 15). Supportive evidence implicating the macrophage was shown by the ninefold decrease in the percentage of macrophages and lymphoid cells after silica treatment. Furthermore, a threefold decrease in the total number of lymphoid cells and macrophages in the peritoneal cavity was associated with decreased resistance to EMCV after i.p. injection of cyclophosphamide, a known T-lymphocyte (16, 19, 22), B-lymphocyte (22, 25), and monocyte-macrophage (2, 8, 26) immunosuppressant. Because our previous data have shown that enhanced resistance of mycobacteria-injected mice to EMCV is not associated with anti-EMCV antibody (12), and therefore B-lymphocytes by association, and experiments reported here have indicated a nonessential role for T-lymphocytes in resistance, it appears that the reduction in macrophage numbers after treatment with silica and cyclophosphamide was associated with decreased resistance to EMCV.

Because nonviable *M. tuberculosis* was effective in thymectomized and nude mice, it would appear that macrophages acted independently of T-lymphocytes in enhancing resistance to EMCV. This finding of macrophage activation in the absence of T-lymphocytes is not novel in that others have shown stimulation of phagocytic activity in T-lymphocyte-deprived mice (27), an increase in resistance to *Listeria* infection in nude mice (20), and generation of cytotoxic activity to tumor cells in T-lymphocyte-deprived and nude mice (9). That activated macrophages were present in our mice was highly probable because Kelly has identified these cells in animals injected with oil-droplet emulsions of *M. bovis* strain BCG cell walls (10). Studies are in progress to identify the peritoneal exudate cells from mice injected with nonviable mycobacteria that inhibit EMCV replication in mouse embryo cells (data not shown) and to determine the means by which this inhibition occurs.

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