Antiviral Research, 7 (1987) 161–167
Elsevier

AVR 00230

Role of interferon, antibodies and macrophages in the protective effect of *Corynebacterium parvum* on encephalomyocarditis virus-induced disease in mice

Monique Géniteau-Legendre, Françoise Forestier, Anne-Marie Quéro and Albert German

Laboratoire de Virologie et d'Immmunologie Expérimentales, Centre d'Etudes Pharmaceutiques – Université Paris XI, 5 rue J.B. Clément, F-92296 Chatenay-Malabry Cedex, France

(Received 2 October 1986; accepted 29 October 1986)

Summary

Treatment of mice with *Corynebacterium parvum* enhances their resistance to encephalomyocarditis (EMC) virus infection. In EMC-virus-infected mice, pretreated with *C. parvum*, neither interferon production nor antibody responses were increased. A decrease of virus recovery was observed in cultures of macrophages taken from the peritoneum of mice early after injection of *C. parvum* and infected with EMC-virus in vitro. The data suggest that *C. parvum* acts by increasing intrinsic antiviral activity of macrophages.

Corynebacterium parvum; Encephalomyocarditis virus; Macrophage

Introduction

Treatment of mice with *Corynebacterium parvum* is known to enhance their resistance to tumor growth (Berd, 1978; Halpern, 1979), bacterial (Adlam et al., 1972; Collins and Scott, 1974; Miyata et al., 1980), protozoal (Nussenzweig, 1967) and some viral infections (Cerutti, 1974; Kirchner et al., 1977a, 1978). In a previous study, we showed a protective action of *C. parvum* against encephalomyocarditis (EMC), herpes and influenza virus infections (Géniteau et al., 1977). Since resistance was most pronounced in experiments using EMC, we have used this virus to study the mechanisms of action of *C. parvum*. Resistance against viral infection is complex and involves multiple mechanisms. Interferon is an important early

component of host resistance to EMC-virus (Gresser et al., 1976). Antibodies also seem to play a role since Murphy and Glasgow showed that the administration of anti-EMC-virus neutralizing antibodies to immunosuppressed mice restored their resistance to infection (Murphy and Glasgow, 1978). Hence, we investigated the effect of treatment with *C. parvum* on interferon and antibody production during EMC-virus infection. Furthermore, it has been suggested that macrophages play a role in natural resistance to EMC virus (Allison, 1974; Dickinson and Griffiths, 1966) and activation of these cells by *C. parvum* is a well-known phenomenon. This led us to also study their role in *C. parvum*-stimulated resistance in our experimental model.

Materials and Methods

In a first experiment we determined EMC-virus levels in serum, brain and spleen of control and *C. parvum*-pretreated EMC virus-infected mice. Groups of three mice (Swiss female, specific pathogen-free, 18–20 g) received single intraperitoneal injections of 700 μg dry weight of *C. parvum* (Wellcome Research Laboratories, Beckenham, Kent, England). Control mice received saline. Two days after this pretreatment the mice were infected with EMC-virus at a dose (2×10^4 $TCID_{50}$) causing about 70% mortality in controls. Previous preliminary

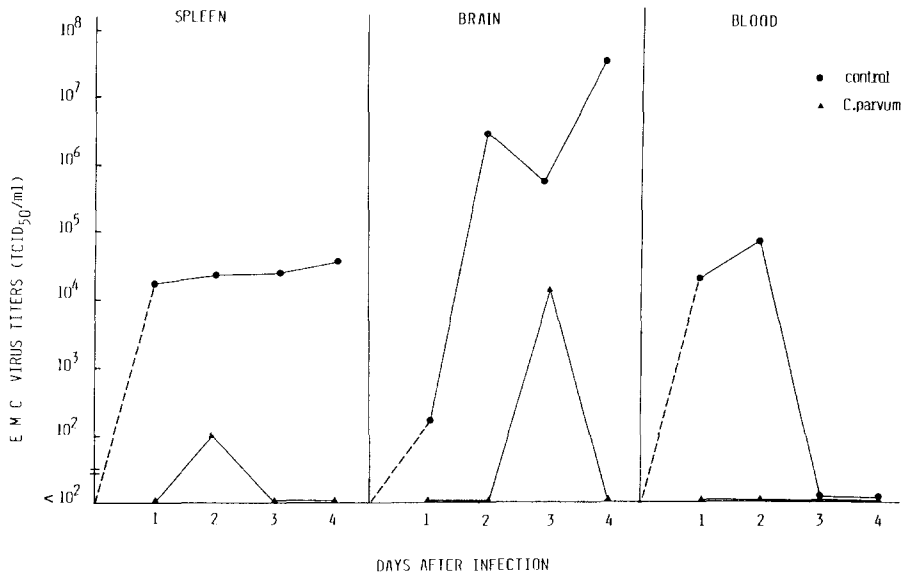

Fig. 1. Effect of pretreatment with *C. parvum* (day 2 before infection) on virus titers in spleen, brain and blood of EMC virus-infected mice. Virus titrations were done by cytopathic effect (c.p.e.) determination on L cells. Titers were calculated using the Reed and Muench method and expressed as Tissue Culture Infectious Doses $_{50}$/per ml ($TCID_{50}$/ml). Each point represents one determination on pooled samples of 3 mice.

experiences showed us that, under these conditions, *C. parvum* pretreatment provides 80% protection ($P<0.001$). Blood, spleens and brains were collected from the mice at days 1, 2, 3 and 4 post-infection. Virus titers were determined in serum and supernatants of brain and spleen homogenates. The results are shown in Figure 1. In the treated group, viral titers were lower than in the control group: virus was never detected in blood samples of *C. parvum*-treated mice. Whereas in spleen and brain samples of control mice virus titers rose progressively, in *C. parvum*-treated mice virus was only transiently detected on days 2 and 3 after infection, with a difference of about 2 $\log_{10}$ compared to controls. Furthermore, lower virus titers in all samples were observed as early as 24 h after infection. A second experiment performed with a lower dose of *C. parvum* (100 μg) gave similar results: 4 days after infection virus titers in spleen and brain samples of *C. parvum*-treated mice were about 1 $\log_{10}$ $TCID_{50}$ lower than in control mice. We conclude that the protective effect of *C. parvum* on EMC-virus infection in mice was associated with decreased virus titers in the blood, spleen and brain. The decrease was observed as early as 24 h after infection, thus suggesting that it was related to a nonspecific increase of host resistance. Therefore, an experiment similar to that described in the previous section was done to determine interferon levels in serum, peritoneal fluid, spleen and brain of EMC virus-infected mice that had been treated with *C. parvum* or saline.

As shown in Table 1 interferon was not detectable in peritoneal fluid of control and *C. parvum*-pretreated mice at any time after infection. In blood, low titers interferon (10 IU/ml) were detected only in control mice from 2 to 4 days after infection. The highest were found in spleens and brains on days 3 and 4, and at these days, the titers of interferon found in brain were lower in *C. parvum*-treated mice than in control mice. In a second experiment, identical to that described in Table

TABLE 1

Effect of pretreatment with *C. parvum* on interferon levels[a] in blood, peritoneal fluid, spleen and brain of EMC virus-infected mice

Time after infection (h)	Interferon levels							
	Blood[b]		Peritoneal fluid[b]		Spleen[c]		Brain[c]	
	Control	*C. parvum*	Control	*C. parvum*	Control	*C. parvum*	Control	*C. parvum*
4	<10	<10	<10	<10	40	80	<10	40
24	<10	<10	<10	<10	10	160	<10	<10
48	10	<10	<10	<10	80	80	160	40
72	10	<10	<10	<10	320	320	160	20
96	10	<10	<10	<10	160	80	640	160

[a] Assays were done by a c.p.e. inhibition assay on L cells using vesicular stomatitis virus as a challenge. Interferon titers were adjusted on a laboratory standard interferon preparation provided by Prof. Poindron (France), that was calibrated against the international standard preparation.

[b] Blood and peritoneal fluid was titrated individually (5 mice for each point); interferon expressed as units/ml.

[c] Interferon was determined on 1/10(w/v) pooled homogenates of spleens and brains (5 mice for each point); interferon expressed as units/gr.

1, interferon was only detected in a single brain sample from control mice infected 4 days previously.

There is no doubt that endogenous interferons play a role in EMC virus infection. Thus, Gresser et al. (1976) showed that treatment of mice with anti-interferon-α/β globulin markedly decreased their resistance to the virus. In accordance with these studies, we found low interferon levels in control mice, the maximal interferon production being observed in the spleen on days 3 and 4 after infection. At that time titers of interferon detected in spleen samples of *C. parvum*-treated mice were identical to that of control mice; in brain samples, titers of interferon were lower in *C. parvum*-treated mice than in control mice. There is also no doubt that *C. parvum* can trigger the interferon system. Thus, Kirchner et al. (1977b, 1978) working with C57Bl/6 mice reported that *C. parvum* induces γ-type interferon production both in vivo and in vitro. In vivo interferon was detectable from day 5–20 following *C. parvum* inoculation with a peak on day 7. However, this production could not be held responsible for resistance enhancement as early as 1 day post-infection as seen in our studies.

Antibodies have also been reported to be an important determinant of host resistance during primary systemic EMC-virus infection (Murphy and Glasgow, 1978; Glasgow, 1970). Since *C. parvum* has been shown to enhance the humoral reponse to various antigens (Berd, 1978; Liacopoulos-Briot et al., 1977; Warr and Sljivic, 1974; Watson and Sljivic, 1976), it was tempting to speculate that the protective effect of *C. parvum* might be due to enhanced production of anti-EMC-virus-an-

TABLE 2

Effect of pretreatment with *C.parvum* on the antibody response of EMC virus-infected mice

Antibody type[b]	Treatment	Days after EMC virus infection											
		4	5	6	7	8	9	11	12	13	14	15	16
Hemagglutination inhibition	Control	64[a]	64	32	256	64	n.d.	32	32	128	64	128	n.d.
	C.parvum	64	64	32	64	32	n.d.	64	256	64	128	256	n.d.
Neutralization	Control	n.d.	35	35	35	35	1780	n.d.	300	2000	>4096	35	35
	C.parvum	n.d.	<4	<4	<4	<4	<4	n.d.	11	<4	<4	<4	<4

[a] Inverse of end-point dilution; n.d. = not determined.

[b] Antibody was determined on pooled sera of 3 mice from each group. The hemagglutination-inhibition test, previously described by Craighead and Shelokov (1961) was used with slight modifications. Inactivated sera were adsorbed with a 50% suspension of sheep red blood cells (SRBC) to remove nonspecific agglutinins and with a 25% suspension of kaolin to remove nonspecific inhibitors. The tested sera were diluted in a 0.05 M borate-buffered 0.12 M KCl solution (pH 8). Four hemagglutinating units of antigen in 50 μl were added to 50 μl of the diluted sera. After incubation at room temperature for 1 h., 50 μl of a 0.2% suspension of SRBC were introduced. The antibody titer was expressed as the reciprocal of the highest dilution causing complete hemagglutination inhibition. The neutralization test was performed by adding a virus inoculum containing 100 $TCID_{50}$/0.1 ml to appropriate dilutions of the tested sera. After 30 min incubation at 37°C of the serum-virus mixtures, the residual virus was titrated by c.p.e. determination on L cells. Antibody titers were expressed as the reciprocal of the highest serum dilution causing 50% c.p.e. reduction.

tibody. Therefore, in an experiment of similar design as the previous one, antibody titrations were performed on pooled serum samples of three mice from each group, sacrificed between the 4th and 16th day after infection. The results of antibody level determinations are summarized in Table 2. Results obtained with the hemagglutination-inhibition method show that there was no significant difference between *C. parvum*-treated mice and control mice. With the neutralization method high levels of antibodies were reached in control mice between the 9th and 14th day, with a peak level >4096, whereas no antibody was detected in *C. parvum*-treated mice. This observation is in keeping with the fact that *C. parvum* antiviral effect was obvious as early as 24 h after infection and that at that time virus titers were lower in *C. parvum*-treated mice than in control mice.

C. parvum is known to be a potent stimulant of the mononuclear phagocyte system (Berd, 1978; Castro, 1974; Mantovani et al., 1976) and there is considerable evidence that macrophages are an important component in host resistance (Mogensen, 1979). Therefore, we determined EMC-virus titers in peritoneal exudates after intraperitoneal inoculation of the virus in *C. parvum*-treated and control mice. Five mice from control and *C. parvum*-pretreated groups were killed at 1, 2, 6, 8 and 24 h after infection. 0.5-ml aliquots of peritoneal exudate were collected after injection of 1 ml of cold phosphate-buffered saline (PBS) in the peritoneal cavity. The pooled peritoneal exudate samples were centrifugated and stored at −70°C. Virus titrations were performed by c.p.e. determination on L cells. As shown in Table 3, in the treated group, virus titers were always at least 2 $\log_{10}$ $TCID_{50}$ lower than in the control group; 24 h after infection the difference was at least 2 $\log_{10}$ $TCID_{50}$.

In a final experiment we examined whether peritoneal macrophages from *C. parvum*-treated mice would possess a lesser ability to replicate or an increased ability to destroy EMC virus. For this type of experiment the peritoneal cavities of 10–15 mice, control or pretreated with *C. parvum* 48 h beforehand, were washed out with cold Hank's solution containing 10 units/ml of heparin. The pooled peritoneal cells were washed twice with Eagle's minimum essential medium (MEM) supplemented with 10% inactivated fetal calf serum and resuspended in the same medium. 10^6 cells were plated in 0.5 ml medium per well of tissue culture plates (Costar Cluster, Poly-Labo, Strasbourg, France). After a 2-h incubation at 37°C, non-adherent cells were removed by three washes with MEM, and 0.5 ml of fresh

TABLE 3

Virus titers[a] in peritoneal exudates of EMC-virus-infected control and *C.parvum*-pretreated mice

	Virus titer ($\log_{10}$ $TCID_{50}$/ml) at time (h.)				
	1	2	6	8	24
Control	2.58[b]	2.32	<2.00	<2.00	4.18
C.parvum	2.20	<2.00	<2.00	<2.00	<2.00

[a] Virus titers determined as indicated in the legend of Fig. 1.

[b] Each figure represents one determination on pooled exudate of 5 mice.

TABLE 4

EMC virus yield in cultures of peritoneal macrophages of control and *C.parvum*-pretreated mice

Dose of virus inoculated[a]	Virus yield (log 10 $TCID_{50}$/ml)				
		0 h	4 h	24 h	48 h
2×10^7	Control	5.24	5.12	3.64	<2.00
	C.parvum	5.50	4.58	2.15	<2.00
2×10^6	Control	4.12	4.30	3.00	2.7
	C.parvum	4.12	3.70	<2.00	<2.00

[a] Triplicate cultures of cells (2×10^6 cells) were infected with the EMC virus. After 1 h absorption period, the cultures were washed and fresh medium was added. Immediately (0 h) and at the indicated times, cells and fluids were harvested, sonified and assayed for virus content (see legend of Fig. 1). Each point represents one determination on pooled samples of 3 mice.

medium was added per well. Adherent cells were maintained for 24 h at 37°C in a 5% CO_2/air atmosphere. The medium was then removed and 0.5 ml inocula of EMC-virus (2×10^6 or 2×10^7 $TCID_{50}$), diluted in MEM with 2% fetal calf serum, were added into each well. After 1 h at 37°C, the residual inoculum was removed by two washes and fresh medium was added immediately and after 4, 24 and 48 h incubation at 37°C, cells were scraped off with a rubber policeman; cell suspensions obtained from 3 wells were pooled and frozen at −70°C until assay after three freezing and thawing cycles. The results, summarized in Table 4, show that virus titers decreased with time, and did so more rapidly in macrophage cultures from *C. parvum*-treated mice than in those for control mice. These results indicate that *C. parvum*-activated macrophages have an increased inactivating effect on EMC-virus. Therefore, it seems likely that macrophages from *C. parvum*-treated mice develop an intrinsic anti-EMC activity which might be due to a greater ability to phagocytize and degrade the virus.

Acknowledgments

We wish to thank Dr. P. Verroust for helpful advice during the writing of this manuscript. We thank Prof. P. Poindron who kindly provided the laboratory standard interferon preparation.

References

Adlam, C., Broughton, E.S. and Scott, M.T. (1972) Enhanced resistance of mice to infection with bacteria following pretreatment with *Corynebacterium parvum*. Nat. New Biol. 235, 219–220.

Allison, A.C. (1974) On the role of mononuclear phagocytes in immunity against viruses. Prog. Med. Virol. 18, 15–31.

Berd, D. (1978) Effects of *Corynebacterium parvum* on immunity. Pharmacol. Ther. A, 2, 373–395.

Castro, J.E. (1974) The effect of *Corynebacterium parvum* on the structure and function of the lymphoid system in mice. Eur. J. Cancer 10, 115–120.

Cerutti, I. (1974) Propriétés antivirales du *C. parvum*. C.R. Acad. Sci. (Paris), Serie D, 279, 963–966.
Collins, F.M. and Scott, M.T. (1974) Effect of *Corynebacterium parvum* treatment on the growth of *Salmonella enteritidis* in mice. Infect. Immun. 9, 863–869.
Craighead, J.E. and Shelokov, A. (1961) Encephalomyocarditis virus hemagglutination-inhibition test using antigens prepared in HeLa cell cultures. Proc. Soc. Exp. Biol. Med. 108, 823–826.
Dickinson, L. and Griffiths, A.J. (1966) The pathogenesis of experimental infection with encephalomyocarditis (EMC) virus. Br. J. Pathol. 47, 35–44.
Géniteau, M., Quéro, A.M. and German, A. (1977) Action de *Corynebacterium parvum* sur différentes infections virales chez la souris. Ann. Pharm. Fr. 35, 181–187.
Glasgow, L.A. (1970) Interrelationships of interferon and immunity during viral infections. J. Gen. Physiol. 56, 212–226.
Gresser, I., Tovey, M.G., Bandu, M.T., Maury, C. and Brouty-Boyé, D. (1976) Role of interferon in the pathogenesis of virus diseases in mice as demonstrated by the use of anti-interferon serum. I. Rapid evolution of encephalomyocarditis virus infection. J. Exp. Med. 144, 1305–1315.
Halpern, B.N. (1975) *Corynebacterium parvum*. Applications in Experimental and Clinical Oncology, pp. 101–401. Plenum Publishing Corp. New York, NY.
Kirchner, H., Hirt, H.M. and Munk, K. (1977a) Protection against herpes simplex virus infection in mice by *Corynebacterium parvum*. Infect. Immun. 16, 9–11.
Kirchner, H., Hirt, H.M., Becker, H. and Munk, K. (1977b) Production of an antiviral factor by murine spleen cells after treatment with *Corynebacterium parvum*. Cell. Immunol. 31, 172–176.
Kirchner, H., Scott, M.T., Hirt, H.M. and Munk, K. (1978) Protection of mice against viral infection by *Corynebacterium parvum* and *Bordetella pertussis*. J. Gen. Virol. 41, 97–104.
Liacopoulos-Briot, M., Lambert, F. and Halpern, B. (1977) The cells involved in the modification of the 'in vitro' immune response by *Corynebacterium parvum*. Dev. Biol. Stand. 38, 215–221.
Mantovani, A., Tagliabue, A., Vecchi, A. and Spreafico, F. (1976) Effect of *Corynebacterium parvum* on cellular and humoral antitumoral immune effector mechanisms. Eur. J. Cancer 12, 113–123.
Miyata, H., Nomoto, K. and Takeya, K. (1980) Characteristics of resistance to *Listeria monocytogenes* enhanced by *Corynebacterium parvum* in mice. Immunology 40, 33–39.
Mogensen, S.C. (1979) Role of macrophages in natural resistance to virus infections. Microbiol. Rev. 43, 1–26.
Murphy, B.R. and Glasgow, L.A. (1978) Factors modifying host resistance to viral infection. III. Effect of whole body X-irradiation on experimental encephalomyocarditis virus infection in mice. J. Exp. Med. 127, 1035–1052.
Nussenzweig, R.S. (1967) Increased nonspecific resistance to malaria produced by administration of killed *Corynebacterium parvum*. Exp. Parasitol. 21, 224–231.
Rabinovitch, M., Manejias, R.E., Russo, M. and Abbey, E.F. (1977) Increased spreading of macrophages from mice treated with interferon inducers. Cell. Immunol. 29, 86–95.
Warr, G.W. and Sljivic, V.S. (1974) Enhancement and depression of the antibody response in mice caused by *Corynebacterium parvum*. Clin. Exp. Immunol. 17, 519–532.
Watson, S.R. and Sljivic, V.S. (1976) The role of macrophages in the adjuvant effect on antibody production of *Corynebacterium parvum*. Clin. Exp. Immunol. 23, 149–153.