Protection of Mice Against Viral Infection by Corynebacterium parvum and Bordetella pertussis

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SUMMARY

Mice could be significantly protected against infection with herpes simplex virus (HSV) by i.p. or i.v. injection of killed Corynebacterium parvum 7 days before infection. This protection was seen in inbred strains of mice with a different degree of sensitivity to HSV and after both i.p. and i.v. infection. Resistant mice immunosuppressed by X-irradiation and showing an increased susceptibility to HSV could also be protected by a previous injection of C. parvum. Elevated levels of interferon were demonstrated in the serum of mice injected with C. parvum 5 to 12 days previously. Four different strains of anaerobic coryneforms were compared and only those which were able to induce a systemic activation of the lymphoreticular system (as reflected by splenomegaly) protected against HSV infection. Protection against HSV-infection could also be demonstrated by using killed Bordetella pertussis. C. parvum also protected against Semliki Forest virus infection in two different strains of mice.

INTRODUCTION

There is increasing evidence to indicate that macrophages play a critical role in the defence against virus infections (Allison, 1974). In certain schedules of infection macrophages appear to represent the first line of defence against the virus. Some viruses such as herpes simplex virus (HSV) are effectively restricted by macrophages which support an abortive infection without replication (Stevens & Cook, 1971). Treatment of mice with silica or anti-macrophage serum has been shown to increase susceptibility to experimental infection with HSV (Zisman et al. 1970). Therefore, it is logical to try to combat such virus infections by activating macrophages. Previously, this approach has been little studied but during the completion of our experiments a few reports have appeared demonstrating that certain immunostimulants which are known to cause a general activation of the lymphoreticular system are effective in protecting mice against experimental virus infections (Starr et al. 1976; Glasgow et al. 1977; Morahan et al. 1977). In the present paper we summarise some of our recent data on the protective effect of Corynebacterium parvum and Bordetella pertussis in HSV-infection of mice.

METHODS

Virus. A strain of HSV-1 designated HSV (WAL) (Kirchner et al. 1977a) was used in all experiments. This strain, which after repeated passage in mouse brain was highly pathogenic
for mice, was re-adapted to tissue culture and was used after the second passage in human embryo lung cells (HEL). A single stock of virus, frozen in small samples at -70 °C was used in all experiments. It contained $7 \times 10^8$ p.f.u./ml when determined by virus plaque assay in cultures of HEL.

Semliki Forest virus (SFV) of the L10 C I strain (Bradish & Allner, 1972) was a gift from Dr C. J. Bradish (Porton Down, Salisbury, U.K.) to Dr V. Schirrmacher. It was passaged once in brains of suckling BALB/c mice.

_Mice._ Male BALB/c/A BOM, C57BL/6/J BOM, and DBA/2/J BOM mice were obtained from Bomholtgard (Rye, Denmark). They were used in the experiments at the age of 8 to 12 weeks. STU mice which originally had been obtained from Dr W. Schäfer (Tübingen, FRG) were bred in our Department by continuous brother-sister mating.

_Bacterial stimulants._ Corynebacterium parvum (CN 6134) was a formalin-killed suspension (7 mg dry weight/ml) from Burroughs Wellcome, Beckenham, Kent, U.K. Other strains of anaerobic coryneforms designated CN 5888, CN 6276 and CN 5936 were similarly prepared (Adlam & Scott, 1973) and provided by Dr C. Adlam of Burroughs Wellcome. Dilutions were made in saline.

Killed _Bordetella pertussis_ prepared by Behringwerke (Marburg, FRG) was provided through the courtesy of Dr Hof (Würzburg, FRG). It also contained thiomersal and the concentration of bacteria in the original suspension was $10^{11}$/ml. It was stored at 4 °C and diluted in saline before use.

_Evaluation of animal experiments._ Groups of 10 to 20 mice were injected with the immunoadjuvants at various times before or simultaneously with the virus infection. The optimal doses were tested in initial experiments and optimal protection was seen with 350 µg _C. parvum_ or $10^8$ organisms of _B. pertussis_ per mouse. Control groups usually received an injection of saline. Various virus doses were also tested in preliminary experiments and significant protection could often be observed after infection with up to 100 LD$_{50}$. Routinely, mice were infected with 20 to 40 LD$_{50}$ as indicated in the tables and figures. The time of death was recorded daily for 20 days after virus infection since it was known that no deaths occurred later than 15 days after infection with HSV or SFV. The percentage of surviving mice in the experimental and control group was compared by the chi square test.

_Determination of serum levels of interferon._ Mice were bled from the retro-orbital sinus and the serum was recovered and diluted in balanced salt solution. The titres of interferon were tested in a plaque-reduction assay using vesicular stomatitis virus and L cells. All details of this assay and of the reference standards used etc. have been described (Hirt _et al._ 1978).

**RESULTS**

**Protection of STU mice by Corynebacterium parvum**

In initial experiments we have used STU mice which are quite susceptible to HSV infection, to determine the conditions for protecting mice against viral infection by _Corynebacterium parvum_. Protection was optimal when _C. parvum_ was given 7 days before HSV. Little or no protection was seen when it was injected 4 days before, or on the day of virus infection. It was equally effective in protecting STU mice against an intraperitoneal (i.p.) infection with HSV when given by the intravenous (i.v.) route as after i.p. dosage (Table 1).
Table 1. Protection of STU mice by treatment with killed Corynebacterium parvum organisms

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Treatment</th>
<th>Timing</th>
<th>No. of mice dead/total no. treated</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>C. parvum i.p.</td>
<td>7 days before HSV†</td>
<td>4/20, 17/20</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>C. parvum i.p.</td>
<td>3 days before HSV</td>
<td>15/20, 18/20</td>
<td>NS‡</td>
</tr>
<tr>
<td>II</td>
<td>C. parvum i.p.</td>
<td>7 days before HSV</td>
<td>2/20, 18/20</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>C. parvum i.p.</td>
<td>2 days before HSV</td>
<td>17/20, 20/20</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>C. parvum i.p.</td>
<td>On the day of HSV infection</td>
<td>16/20, 17/20</td>
<td>NS</td>
</tr>
<tr>
<td>III</td>
<td>C. parvum i.v.</td>
<td>7 days before HSV infection</td>
<td>2/20, 17/20</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* 350 μg C. parvum (CN 6134).
† 5 × 10³ p.f.u. (~ 20 LD₅₀ after i.p. infection).
‡ Not significant.
§ 2 × 10⁴ p.f.u. (~ 20 LD₅₀ after i.v. infection).

Table 2. Protection of mice of different strains against infection with HSV by Corynebacterium parvum and Bordetella pertussis given 7 days before infection

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Strain of mice</th>
<th>Treatment</th>
<th>Dose of HSV</th>
<th>No. of mice dead/total no. of mice</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>DBA/2</td>
<td>C. parvum</td>
<td>3 × 10⁴ p.f.u.* i.p.</td>
<td>18/40, 37/40</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>II</td>
<td>BALB/c</td>
<td>C. parvum</td>
<td>5 × 10³ p.f.u.+ i.v.</td>
<td>3/20, 16/20</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>III</td>
<td>C57BL/6</td>
<td>C. parvum</td>
<td>5 × 10³ p.f.u.+ i.p.</td>
<td>0/20, 10/20</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>IV</td>
<td>C57BL/6</td>
<td>C. parvum</td>
<td>1 × 10⁷ p.f.u. i.p.</td>
<td>3/20, 18/20</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>V</td>
<td>C57BL/6</td>
<td>C. parvum</td>
<td>1 × 10⁵ p.f.u. i.p.</td>
<td>2/20, 17/20</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>VI</td>
<td>DBA/2</td>
<td>B. pertussis</td>
<td>1 × 10⁸ p.f.u. i.p.</td>
<td>2/20, 20/20</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>VII</td>
<td>C57BL/6</td>
<td>B. pertussis</td>
<td>1 × 10⁵ p.f.u. i.p.</td>
<td>0/20, 18/20</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

* 20 LD₅₀ in DBA/2 mice after i.p. infection.
† 50 LD₅₀ in BALB/c mice after i.v. infection.
‡ The LD₅₀ for C57BL/6 mice after i.p. infection with HSV (WAL) is 1.2 × 10⁴ p.f.u.
§ 400 R of X-irradiation on the day of virus infection.
|| 10⁸ killed bacteria were injected 7 days before HSV.

Protection of strains of mice other than STU by Corynebacterium parvum against HSV infection

DBA/2 mice could also be protected against i.p. infection with HSV (Table 2). This protection was in most instances less impressive than that observed with STU mice. Clearly, however, the mean number of surviving mice pre-treated with C. parvum was significantly higher than that of the controls, pre-treated with saline.
BALB/c mice which, in contrast to other mouse strains, are quite susceptible to i.v. infection with HSV (Kirchner et al. 1978) were used to show protection by an i.p. injection of \textit{C. parvum} against this schedule of HSV infection (Table 2).

It was previously shown that C57BL/6 mice, which are relatively resistant to i.p. infection with HSV, could be killed by high virus doses (Kirchner et al. 1978). A good protective effect was seen when \textit{C. parvum} was given 7 days before infection of C57BL/6 mice with high doses of HSV. X-irradiation (400 R) when applied on the day of HSV infection to C57BL/6 mice markedly decreased the LD$_{50}$, but these immunodepressed C57BL/6 mice could still be protected by \textit{C. parvum}, given 7 days before the virus (Table 2).

\textit{Protection of DBA/2 mice against HSV infection by Bordetella pertussis}

DBA/2 and C57BL/6 mice could be well protected against HSV infection by a previous injection of \textit{B. pertussis} (Table 2). As with \textit{C. parvum}, protection was seen only when the bacteria were injected 7 days before the virus but not when they were given 3 days before injection. The optimal dose for protection was $10^8$ organisms per mouse and protection could be observed with doses of HSV up to 100 LD$_{50}$ (data not shown).

\textit{Measurement of serum interferon levels}

C57BL/6 mice were injected i.p. with 350 $\mu$g \textit{C. parvum} and serum interferon levels were determined at various times thereafter. As can be seen in Fig. 1, significant levels of interferon could be detected between 5 and 20 days after injection of \textit{C. parvum}. Little or no interferon was found earlier than 5 days.

\textit{Testing of different strains of coryneform bacteria}

Four different strains of anaerobic coryneforms (selected by Dr C. Adlam for their differing spleen weight stimulating abilities) were provided in coded form and tested blind for their ability to induce splenomegaly and to protect DBA/2 mice against subsequent HSV infection (Table 3). Only the two bacterial strains which were able to increase the spleen weight were protective against HSV infection.

![Graph showing serum interferon levels after injection of \textit{C. parvum}.]
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Table 3. Comparison of the lymphoreticular stimulating ability of various strains of anaerobic coryneforms with their ability to protect DBA/2 mice against HSV infection*

<table>
<thead>
<tr>
<th>Anaerobic coryneform strain</th>
<th>Spleen index†</th>
<th>No. of mice dead/total no. of mice</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN 6134</td>
<td>8.2</td>
<td>4/20</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>CN 5888</td>
<td>1.0</td>
<td>20/20</td>
<td>NS‡</td>
</tr>
<tr>
<td>CN 6276</td>
<td>1.5</td>
<td>18/20</td>
<td>NS‡</td>
</tr>
<tr>
<td>CN 5936</td>
<td>6.8</td>
<td>9/20</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Controls injected with saline</td>
<td>1.0</td>
<td>20/20</td>
<td></td>
</tr>
</tbody>
</table>

* 350 g of the appropriate bacterial strain were injected i.p. and 7 days later mice were challenged with 50 LD50 of HSV i.p.
† 350 g of the bacteria were injected i.v. and 14 days later spleens were removed and weighed. Spleen index: ratio of spleen weights of injected mice compared with saline injected mice.
‡ In comparison with the saline group; NS, not significant.

Table 4. Effect of previous irradiation of C57BL/6 mice on the protection by Corynebacterium parvum against HSV infection*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice dead/total no. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Day 0 10/10</td>
</tr>
<tr>
<td>Group II</td>
<td>Day 7 0/10</td>
</tr>
<tr>
<td>Group III</td>
<td>Day 0 0/10</td>
</tr>
<tr>
<td>Group IV</td>
<td>Day 7 0/10</td>
</tr>
<tr>
<td>Group V</td>
<td>Day 0 10/10</td>
</tr>
<tr>
<td>Group VI</td>
<td>Day 7 8/10</td>
</tr>
</tbody>
</table>

* Experimental conditions as in Expt. IV of Table 2.
† The difference between group II and V was significant (P < 0.05) while the difference between group II and VI was not.

Table 5. Protection of C57BL/6 and DBA/2 mice against i.p. infection with Semliki Forest virus*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mouse strain</th>
<th>No. of mice dead/total no. of mice</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corynebacterium parvum†</td>
<td>C57BL/6</td>
<td>1/20</td>
<td>0.01</td>
</tr>
<tr>
<td>Saline</td>
<td>C57BL/6</td>
<td>20/20</td>
<td></td>
</tr>
<tr>
<td>C. parvum</td>
<td>DBA/2</td>
<td>2/20</td>
<td>0.01</td>
</tr>
<tr>
<td>Saline</td>
<td>DBA/2</td>
<td>19/20</td>
<td></td>
</tr>
</tbody>
</table>

* Mice were challenged with 100 LD50 of SFV.
† 350 g C. parvum per mouse, 7 days before infection.

Effect of previous X-irradiation on the protective effect of Corynebacterium parvum on HSV infection

X-irradiation (400 R) given 4 days before C. parvum abolished the protective effect of C. parvum on HSV infection of C57BL/6 mice (Table 4). However, no such adverse effect was seen when the radiation dose was applied 3 days after C. parvum (i.e. 4 days before virus infection).
Protection of C57BL/6 and DBA/2 mice against SFV by injection of Corynebacterium parvum

In contrast to an i.p. infection with HSV (Lopez, 1975) C57BL/6 and DBA/2 mice were found to be equally susceptible to i.p. infection with SFV (Table 5). Both strains of mice could be well protected by injection of C. parvum given 7 days before the virus.

DISCUSSION

The genetic resistance of mice to mouse hepatitis virus is determined by a difference in the ability of macrophages from different mouse strains to replicate this virus (Bang & Warwick, 1960). Genetic differences in the susceptibility of mice to HSV infection have been also reported (Lopez, 1975). However, thus far there is little indication that HSV replicates in macrophages (Stevens & Cook, 1971; Kirchner et al. 1976) and other reasons have to be sought to explain the genetic resistance of certain mouse strains to HSV. In this regard, it seems to be of interest that susceptible strains of mice could be equally well protected against HSV infection by Corynebacterium parvum as C57BL/6 mice which are relatively resistant.

The basis of the protective effect of C. parvum on a virus infection has not been fully understood. However, protection is obviously more than just a local effect at the injection site since protection was also seen when C. parvum was given by a route other than that of virus infection. C. parvum has been shown to induce a strong systemic activation of the lymphoreticular system (Scott, 1974) which appears to be caused by mobilization of macrophage precursors from the bone marrow into the periphery (Baum & Breese, 1976). Not all strains of coryneforms are active in this regard and it is noteworthy that those which were inactive (i.e. did not induce splenomegaly) also caused no antiviral protection. Woodruff et al. (1976) have shown that the lymphostimulatory effects of C. parvum in mice could be prevented by previous X-irradiation. They have suggested that the effect of C. parvum on the macrophage system is, to a considerable extent, due to the stimulation of radiosensitive macrophage precursors to differentiate into mature cells which are actively phagocytic and cytotoxic. Our data are in agreement with their findings since we have observed that the antiviral effects of C. parvum could be abolished by previous irradiation.

Previously, it has been shown that mice injected with Corynebacterium acnes (which is in most properties closely related to C. parvum) displayed a reduced capacity to respond to interferon inducers (Farber & Glasgow, 1972). However, we have found that spleen cells of mice injected in vitro with C. parvum elaborated increased levels of interferon (Kirchner et al. 1977b). Here we have found that significant levels of interferon could also be found in the serum of C. parvum-treated mice.

The data from Glasgow's laboratory and ours are not necessarily at variance with each other. The interferon produced by spleen cells from C. parvum-injected mice is produced only 5 to 10 days after injection. This markedly contrasts with conventional induction of interferon, such as performed by Farber & Glasgow (1972), where high levels of interferon are detected in the serum several hours after injection of the inducer. In interferon research it is known that 'blocking' occurs when cells which are responding to one type of interferon inducer are challenged with a second inducer (Ho & Armstrong, 1975). This may occur when the cells of the lymphoreticular system which are already responding to C. parvum are challenged with another inducer.

It is clear, however, that at the present stage we cannot prove that induction of interferon is the cause of the antiviral effect of C. parvum. Furthermore, although it is quite likely that
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Macrophages play a significant role in this protection, their definitive role also remains to be determined.

Bordetella pertussis is known to induce a lymphocytosis in mice (Morse & Riester, 1967) but its effects as an activator of macrophages have been less well studied. In experimental tumour work, divergent results have been reported. In some studies B. pertussis was able to protect against a tumour (Likhite, 1974), whereas in others there was little protection (Purnell et al. 1975) or even an adverse effect (Floersheim, 1967). In our studies, B. pertussis also protected mice against HSV infection, and protection was observed against high virus doses and in a mouse strain where protection by Corynebacterium parvum was quite weak. The basis for this protective effect of B. pertussis needs to be clarified by further experiments, but it seems of interest that B. pertussis also has been shown to be an interferon inducer (Borecky & Lackovic, 1967).

Lately, a number of immunosuppressive effects of the so-called ‘immunostimulants’ have been noted (Scott, 1972; Kirchner et al. 1975). The term ‘immunomodulant’ has been proposed to indicate that these agents may act on a complicated balance between immunostimulation and immunosuppression. From these observations it seems reasonable to conclude that all of these agents should be handled with care since they may be potentially immunosuppressive. However, it remains to be determined which of these various immune functions contribute to host defence. Protection against a virus infection seems to represent true immunopotentation. Corynebacterium parvum is being considered for immunotherapy of human tumours. Since virus infections are a common cause of complications in tumour patients, the antiviral effects of C. parvum may be of great clinical significance.

We thank Mr D. Baumgartl for his co-operation in the animal experiments and Ms B. Mannes for excellent secretarial assistance. Semliki Forest virus was a gift from Dr C. J. Bradish and Bordetella pertussis was kindly provided by Dr H. Hof, University of Würzburg (FRG).

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