Antiviral Activity of an Ether-Extracted Nonviable Preparation of Brucella abortus

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Extraction of living Brucella abortus (strain 456) with aqueous ether yielded a nonviable, insoluble residue (Bru-Pel). When injected into mice, Bru-Pel was an effective, nontoxic interferon stimulus. Mice pretreated with Bru-Pel were protected against challenge with otherwise lethal doses of Semliki Forest virus. Significant protection was afforded when Bru-Pel was given as many as 7 days before virus challenge. Evidence is presented dealing with the complex nature of Bru-Pel and with the possibility that the antiviral activity of Bru-Pel may be associated not only with the production of interferon, but with a general increase in the level of nonspecific resistance in animals.

In 1964, Stinebring and Youngner demonstrated that the intravenous injection of living cells of Brucella abortus into mice induced high titers of interferon in the circulation (16). They reported that peak serum levels of interferon appeared 6 to 8 h after injection of bacteria, a characteristic indicative of the so-called “virus type” of interferon response (18, 21). More recently, Billiau and coworkers extended these results by showing that brucellae rendered nonviable by heating were also capable of inducing interferon production in mice (2). We have now found that Brucella cells extracted with aqueous ether yield a relatively nontoxic preparation (Bru-Pel) that can induce the viral-type interferon response in mice. In addition, Bru-Pel protects the animals against otherwise lethal infection with Semliki Forest virus (SFV). The data to support these conclusions and a description of some properties of Bru-Pel are the subject of the present report.

MATERIALS AND METHODS

Cultivation of bacteria. B. abortus 456 (ATCC 7705), a nonpathogenic strain used for antigen preparation, was grown in tryptose broth with thiamine (Difco) supplemented with 5% calf serum (inactivated at 56°C for 30 min). This strain of Brucella does not require added CO₂ for growth.

One liter of medium in a 2-liter baffled Erlenmeyer flask was inoculated with about 5 x 10⁸ viable Brucella and incubated at 37°C for 48 h with continuous shaking. The growing cells were harvested near the end of the log phase, and viable counts were done in the usual manner on tryptose agar with thiamine and 5% inactivated calf serum.

Preparation of ether-extracted Brucella (Bru-Pel). The bacteria were harvested by centrifugation at 5,000 rpm (4,100 x g) and washed once by suspension in cold, sterile, distilled water and centrifugation. They were then suspended in cold water to give a Klett (filter no. 66) reading of 470 to 500, which represented a viable count of 1 x 10⁶ to 2 x 10⁷ bacteria per ml. (This and all subsequent operations were performed in sterilized equipment. Sterile distilled water was used throughout.) The aqueous suspension was treated with diethyl ether by the methods of Badakhsh and Foster (1) and Ribi et al. (15). Two volumes of cold diethyl ether was added to 1 volume of the cold aqueous suspension of Brucella, and the mixture was shaken for 60 s in a separatory funnel and then allowed to stand at 25°C overnight. The ether layer was discarded and the aqueous phase containing the extracted bacteria was retained. Nitrogen was bubbled through the aqueous cell suspension until the odor of ether was undetectable. The extracted cells were removed by centrifugation, washed with distilled water, and heated at 100°C for 5 min in a water bath to destroy the viability of any bacteria that may have survived the extraction process. This heating did not change the biological activity of the material. The extracted bacteria were recovered by lyophilization of the aqueous heated suspension to yield Bru-Pel. Bru-Pel had the following gross composition (percentage by weight): protein (11), 69%; total carbohydrate as glucose equivalents (14), 10%; fatty acids (10), 12%; and nucleic acid (3), 4%.

The aqueous material remaining after removal of the bacteria contained a significant amount of crude Brucella lipopolysaccharide (LPS). Some properties of this LPS have been described elsewhere (9).

Animals. Female Swiss-Webster mice weighing 25 to 30 g were obtained from Taconic Farms, Germantown, N.Y.

Interferon production and assay. Mice were injected either intravenously in the tail vein (0.1 ml) or intraperitoneally (0.5 ml) with test materials diluted in phosphate-buffered saline (pH 7.0). Bleedings were carried out by cardiac puncture with heparinized...
Interferon assays were done by the plaque reduction method by using a continuous line of mouse L cells (clone 929) and vesicular stomatitis virus as the challenge virus (20). The titers of viral inhibitor were determined by plotting on probit paper the percentage of inhibition against the different dilutions and were expressed as the reciprocal of the dilution of the sample that reduced the plaque count to 50% of the control plaque count. A standard reference interferon sample was included in each assay; this standard varied within a twofold range. One unit of interferon in these assays was the equivalent of 2 units of a reference mouse interferon standard provided by the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases.

SFV. Stock virus was prepared from the brains of infected mice. A 10% suspension of infected brain was clarified by centrifugation and stored at -70°C in small portions. SFV was assayed by the plaque method in primary chicken embryo fibroblasts. Mice were challenged with an appropriate number of plaque-forming units (PFU) in 0.5 ml intraperitoneally; deaths were recorded twice a day for 14 days, after which no further deaths occurred. Statistical significance was determined by the Student t-test criterion.

RESULTS

Interferon production by Bru-Pel in mice. The intravenous or intraperitoneal injection of Bru-Pel into mice resulted in the appearance of a viral inhibitor in the circulation (Table 1). The properties of the viral inhibitor were consistent with those described as characteristic for type I mouse interferons (19). Peak titers occurred 5 to 8 h after injection, and as little as 20 μg of Bru-Pel produced significant amounts of interferon. When Bru-Pel (2,000 or 200 μg) was injected intranasally or intramuscularly into mice, interferon was detected neither in the circulation nor in extracts of lung tissue, nor in muscle from the site of inoculation.

Bru-Pel was not toxic for mice; the intraperitoneal inoculation of as much as 5 mg per mouse, which represented a dose of 200 mg per kg of body weight, produced no deaths or apparent illnesses.

Heating of Bru-Pel in distilled water at 56°C for 4 h or at 100°C for 30 min did not diminish its interferon-inducing ability. On the other hand, this activity could be destroyed by heating Bru-Pel at 100°C for 2 h in water or by heating for 20 min at 100°C in either 0.02 N sulfuric acid or in 0.05 N acetic acid.

Lipids were extracted from Bru-Pel with cold chloroform-methanol (2:1, vol/vol) by the procedure of Oliver and Colwell (13), and lipid-free Bru-Pel as well as the extracted lipids were tested separately for interferon-inducing ability. A dose of 200 μg of lipid-free Bru-Pel, injected intravenously, produced interferon titers of 250 and <32 at 2 and 5 h, respectively. In contrast, the same dose of unextracted Bru-Pel produced interferon titers of 500 and 2,200 at 2 and 5 h. Reconstitution of the extracted Bru-Pel and the lipid recovered from the chloroform-methanol extract did not restore the activity of the starting material; only a small 2-h peak of interferon was observed after injection of the reconstituted material. Parenthetically, it should be noted that lipid-free Bru-Pel did not protect mice from the lethal effects of SFV. These results are in contrast to those obtained with unextracted material, as will be described below.

When mice were pretreated with 244 mg of cycloheximide per kg and injected intravenously with Bru-Pel, the interferon titers at different times did not differ significantly from those obtained without cycloheximide treatment (Table 1). These findings differ from those obtained with Brucella LPS (9). In the latter case, interferon response was significantly enhanced by pretreatment of mice with cycloheximide. These results may reflect the complex nature of the inducer present in Bru-Pel.

Sonic oscillation progressively inactivated the interferon-inducing ability of Bru-Pel. Treatment at 9,000 cycles/s in a Raytheon 200-W, 10-kc magnetostriective oscillator for 1 h produced a 5- to 10-fold loss of interferon-stimulating ability of Bru-Pel; 2 h of sonic treatment essentially abolished all activity. These results are similar to those of Billiau and coworkers (2), who reported that after 2 h of sonic oscillation, the interferon-producing ability of live or heat-killed B. abortus was completely destroyed.
Effect of Bru-Pel on SFV infection of mice.

Two doses of virus were used to challenge mice: 750 and 7.5 PFU per mouse, injected intraperitoneally in 0.5 ml of diluent. The data in Table 2 show that 2,000 or 200 μg of Bru-Pel injected intraperitoneally 24 h before infection with SFV afforded significant protection against the lethal effects of the virus. The average day of death in the control animals was 7.8 and 9.7 days for the high and low doses of virus, respectively. In the groups of mice treated with Bru-Pel, the animals that succumbed did so 1 day later. When 2,000 or 200 μg of Bru-Pel was given as a single dose 6 h before infection with SFV, the same degree of protection was afforded the mice.

The influence of multiple doses of Bru-Pel on SFV infection in mice was also tested. When three 500-μg doses of Bru-Pel were given starting 1 day before infection and 2 and 4 days after infection with SFV, the animals were significantly protected against the lethal effects of the virus. The multiple-dose schedule, starting Bru-Pel treatment 1 day before infection, was no more effective than a single 500-μg dose of Bru-Pel given 24 h before the virus.

An experiment was carried out to determine the protective effect of Bru-Pel treatment started after SFV infection. Mice were injected with 500 μg of Bru-Pel 1 day after SFV infection and again at 2 and 4 days after infection. The control mice were given 0.5 ml of phosphate-buffered saline intraperitoneally on the same schedule. Bru-Pel provided statistically significant protection only against the low dose (7.5 PFU) of challenge virus. With this dose of virus, 24/30 (80%) of the control animals died within 14 days; in the treated mice the mortality was 16/30 (53%, \( P < 0.05 \)). No significant protection occurred when the high dose of virus was used.

A single 500-μg dose of Bru-Pel given to mice 7 days before challenge with SFV produced significant protection against the lethal effects of both the high and low doses of virus (Table 3). Since circulating interferon is not demonstrable in mice 7 days after the injection of Bru-Pel, the protective effect of Bru-Pel under the conditions of this experiment raises some interesting questions concerning the mechanism of its antiviral action.

It is of interest that Kern and his colleagues have found that our preparation of Bru-Pel protected mice against infection with encephalomyocarditis virus and herpesvirus hominis type 2 (personal communication).

**DISCUSSION**

The ability of *B. abortus* to stimulate interferon production and to protect mice against the lethal effects of SFV is retained after the organisms are extracted with ether. Other workers have shown that brucellae extracted with such agents as phenol, ethyl alcohol, or trichloroacetic acid (2, 5) lost all or most of their ability to produce interferon in mice.

The aqueous ether extraction of *Brucella* removed a large proportion of the LPS present in the cell wall (1, 15). When the LPS extracted with ether was purified and injected intravenously into mice, it produced an endotoxin type of interferon response, with peak titers of inhibitors appearing in the circulation at 2 h (9). One might argue that in the animal the slow release of residual *Brucella* LPS from Bru-Pel may give rise to the viral type of interferon response (5- to 8-h peak) observed in our experiments. This view, however, seems to be inconsistent with a number of observations. First, Bru-Pel lost all interferon-inducing ability when heated for 2 h at 100 C, whereas the ability of LPS to produce interferon was unaffected by such treatment. Second, disruption by 2 h of sonic oscillation

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<td>750 PFU</td>
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<td>No. %</td>
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<td>Controls (phosphate-buffered saline at -24 h)</td>
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<td>Bru-Pel</td>
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* Statistical significance: \( P < 0.001 \).

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<td>Controls (phosphate-buffered saline at -7 days)</td>
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<td>Bru-Pel (500 μg at -7 days)</td>
<td>13/30 43*</td>
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* \( P < 0.005 \).

TABLE 2. Protection of mice against SFV by Bru-Pel given 24 h before virus infection

TABLE 3. Protection of mice against SFV by a single dose of Bru-Pel given 7 days before virus infection
essentially eliminated the ability of Bru-Pel to induce interferon; similar results were obtained with viable organisms (2). On the other hand, the same treatment enhanced the ability of LPS to stimulate interferon production (6). Third, the failure of pretreatment of mice with cycloheximide to affect interferon induction by Bru-Pel suggests that LPS is not responsible for the activity of the ether-extracted organisms. The characteristic enhanced interferon response to LPS in mice pretreated with cycloheximide (9, 21) was not seen when Bru-Pel was used as the stimulus.

The nature of the chemical moiety of Bru-Pel responsible for its activity remains unknown. There is no doubt that after ether extraction, which removes much LPS and probably other constituents (1, 15), a complex, organized cell structure remains. Our attempts to identify the active component have not been fruitful. Spheroplasts prepared by the penicillin-glycine method (7), or cytoplasmic membranes of Brucella (8), had no interferon-inducing ability. In addition, interferon-inducing material was not present in cytoplasm recovered from disrupted brucellae (unpublished data). Activity seemed to depend on the maintenance of the structural integrity of the cell. These characteristics lead to the conclusion that the active factor of Bru-Pel resides in some structural component of the Brucella cell itself. It is conceivable that Brucella organisms contain a structure different from that found in other bacteria. One piece of supporting evidence for this possibility is that no other gram-negative bacterial species, including the closely related Yersinia enterocolitica and Pasteurella multocida, produced the virus-type interferon response seen in mice given Brucella (18; Keleti et al., unpublished results). All other gram-negative species tested, living or heat killed, produced a 2-h peak of circulating interferon in mice injected intravenously, the typical endotoxin type of interferon response.

The mechanism by which pretreatment with Bru-Pel increases the resistance of mice to SFV is also not clear. The significant reduction of mortality recorded when Bru-Pel was given to mice as many as 7 days before infection with SFV raises the possibility that the antiviral activity of Bru-Pel may be associated not only with the production of interferon but, perhaps more importantly, with the general increase in the level of nonspecific resistance in animals.

Some support for this hypothesis comes from experiments carried out by others using living and heat-killed Brucella organisms. Billiau et al. (2) reported that mice given living brucellae intravenously were able to resist infection with vaccinia when challenged with the virus as long as 1 month after bacterial infection; heat-killed organisms were much less effective. Muyembe et al. (12) found that mice infected with live B. abortus were protected against Mengo virus infection for at least 3 weeks. Of additional interest is the finding of de Clercq and de Sommer (4) that living B. abortus failed to induce circulating interferon when injected intravenously in rabbits yet effectively protected the animals against local vaccinia virus infection. The severity of infection was reduced whether the living bacteria were injected 1 or 8 days before the virus challenge. More recently, Veskova et al. (17) have reported that the injection of living B. abortus (vaccine strain 19BA) into mice prolonged the survival of animals given Rauscher leukemia virus 1 day earlier. The protection was less effective when the animals were infected with living bacteria before being challenged with the leukemia virus. Again, heat-killed organisms were much less effective than living bacteria. The authors speculate about several different nonspecific mechanisms which could account for the resistance to leukemia virus.

In contrast to the references to the protective effects of living brucellae, Bru-Pel, a nonviable and nontoxic preparation, is capable of protecting mice against the lethal effects of viruses.

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LITERATURE CITED


