Protection of Mice Against Vaccinia Virus by Bacterial Infection and Sustained Stimulation with Specific Bacterial Antigens

EMMA G. ALLEN AND STUART MUDD

Department of Microbiology and Immunology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203 and Philadelphia General Hospital and the Veterans Administration Hospital, Philadelphia, Pennsylvania 19104

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In these experiments, mice which have a strong delayed-type hypersensitivity to mycobacteria were found, when elicited with old tuberculin, to be more resistant to intravenous vaccinia virus challenge than controls. This was manifest as protection from killing when large amounts of virus were injected, or as significantly less tail swelling and damage as well as lower titers of infectious virus when a lesser inoculum was used. Preliminary experiments indicate that animals sensitized with Staphylococcus aureus and elicited with phage lysate of staphylococcus are also more resistant to vaccinia infection.

It is now generally accepted that many living bacterial vaccines provide increased specific resistance to both natural and experimental infection. Living rather than killed vaccines appear to be more efficacious, but the mechanisms of acquiring and maintaining enhanced resistance still are not well understood (4). It has been demonstrated experimentally that, in addition to enhanced specific resistance, infection with mycobacteria also tends to increase nonspecific resistance; that is, the host also exhibits greater resistance to infection with a variety of other bacteria as well as mycobacteria (1, 9). Further work, however, suggests that some nonspecific change does have a specific immunological basis: induction of cell-mediated type hypersensitivity by infection followed by sustained stimulation with specific antigen (elicitation) (9, 10).

The studies of enhancement of nonspecific host resistance have for the most part utilized unrelated bacteria which are found intracellularly in the host, though primary tuberculosis has also been reported to increase resistance of male mice to the lethal effects of ectromelia virus (6); however, these authors state "...it is not known whether this increased resistance to a virus infection results from the same nonspecific immune processes which operate against the bacterial infections." The present studies are a preliminary investigation in attempting to determine whether the mechanisms of bacterial infection and elicitation do indeed extend to the viruses. One might expect this to be so, for macrophages seem to play a key role in both virus infection and host sensitivity. The outcome of cell-mediated resistance involves committed lymphocytes which in the presence of specific antigen can influence macrophage function in various ways; also, the outcome of virus infections may be determined by macrophages which are in excellent locations to monitor viruses in the circulation as well as their entry into various organs (13, 19). These experiments do demonstrate some increase in resistance to vaccinia virus after sensitization with mycobacteria. They also demonstrate a further increase in resistance of sensitized mice after stimulation with specific antigen, this being statistically greater than any conferred by antigen or sensitization alone. Further experiments are in progress to determine what cells or mediators, or both, may be involved.

MATERIALS AND METHODS

Random-bred female albino mice weighing about 30 g (after the period of sensitization) were used in these experiments. Sterile, nonpyrogenic saline was used in preparation of all material for injection.

Sensitization. With Mycobacterium tuberculosis: strain H37Ra organisms were washed off Lowenstein slants in saline, adjusted in turbidity to about 10⁷ organisms per ml, and injected in 0.1-ml amounts into the peritoneal cavity on two occasions with a 2-
week interval between. Such mice were found to be hypersensitive to old tuberculin (OT) by the footpad test, the swelling having a 24- to 48-hr maximum. Mice were used within a 7- to 14-day period after the last sensitizing injection.

With *Staphylococcus aureus*: strain 18Z organisms were grown in Trypticase soy broth with stirring for 18 hr at 36 to 37°C. The organisms were centrifuged at 5,000 rev/min for 30 min in a Sorvall centrifuge at 4°C. The pellets were washed once in one-half volume of sterile saline containing 1% Trypticase soy broth; the cells were then suspended to a concentration of 10^8 viable units per ml, as determined by plate counts. The sensitizing dose was 10^6 units given in 0.1 ml by the subcutaneous route at weekly intervals for a period of 8 weeks. The mice were used within 7 to 14 days after the last sensitizing dose.

**Elicitation.** Specific elicitation of mice sensitive to tube bacillus was carried out by using 1:500 dilution of OT (Parke, Davis & Co.) given intraperitoneally in 0.1-ml amounts 48 hr and 3 to 5 hr before vaccinia challenge.

Specific elicitation of mice sensitized to staphylococcus was carried out by using 0.1 ml of staphylococcal phage lysate (SPL) (Staphage lysate, Delmont Laboratories, Swarthmore, Pa.) given intraperitoneally 24 or 48 hr and 3 to 5 hr before vaccinia challenge.

**Vaccinia challenge.** Mice can be infected with vaccinia by tail vein inoculation (19). Suitable dilution of a 20% allantoic membrane suspension of IHD-T virus (Wyeth Laboratories) was injected in 0.2-ml amounts into the tail veins of mice. Virus stocks were stored in sealed vials in a dry ice chest.

In all experiments the four groups of mice inoculated were alike with respect to sensitization period, age, sex, and size.

Measurements of tail diameter and footpads were made with round, flat calipers having a dial gauge calibrated to 0.05 mm (der Schmittäster, H. C. Kroplin, Deutschland).

**Vaccinia titrations.** The amount of infectious virus was determined by inoculation of tissue suspensions (10%, w/v) on the chorioallantoic membranes of 12-day-old fertile hen eggs. Tissues for inoculation were homogenized in a Waring blender with saline containing penicillin and streptomycin. Each tissue suspension used was checked for bacterial contamination by inoculation of a tube of sterile thioglycolate broth; in no case was bacterial growth observed. After 3 days of incubation at 36 to 37°C, membranes were removed from the eggs and transferred to petri dishes containing saline and 0.5% Formalin, and the poxcs were counted. No less than 12 eggs were used in the calculation of the titer of a given tissue suspension.

**RESULTS**

The amount of swelling or the number of lesions associated with mouse tails after intravenous challenge with vaccinia has been used as indication of the amount of virus growth (2). It was previously reported by Mudd, Zappasodi, and Taubler (Bacteriol. Proc., p. 70, 1969) that infection of mice with H37Ra and elicitation with OT result in significantly less tail swelling after vaccinia challenge when compared with controls. Three present experiments confirmed such differences as can be seen in Fig. 1. Each line represents the average daily changes in tail diameters of 50 mice. In all experiments, normal mice given OT or H37Ra-infected mice averaged slightly less swelling than normal mice; differences between the normal groups were not significant on any day, whereas the H37Ra sensitization further increases resistance which was statistically different from normal mice on day 4. On the other hand, mice sensitized with H37Ra and given OT exhibited significantly less swelling on days 4 through 7 than normal mice and were statistically different at the 0.05 level of significance.

Von Pirquet (22, 23) proposed that the vaccinal lesion is a hypersensitivity response of the host. Others have presented data which support this idea (16). The question which arises concerning the present experiments is whether protection against vaccinia afforded by infection and elicitation reflects decreased vaccinia replication or decreased ability on the part of the host to develop or show sensitivity to vaccinia. In an effort to answer this, infectivity titrations of mouse tissues were carried out at different times after virus challenge.

The first tissue examined was the tail, since the tail is an obvious site of virus damage. Titrations of tail tissues (pools of five tails) were taken at the peak day of swelling. These titers are given in Table 1. There was less virus in tails having less swelling: tails of H37Ra-infected mice which had been given OT contained less infectious virus than tails of normal animals given OT, there being a 3- to 18-fold difference in virus titers, which is significant at 0.01 level. The amount of virus present in tails of sensitized mice was not significantly different from that in tails of normal mice receiving OT.

Enhancement of nonspecific host resistance has been shown by others not to be restricted to infection with mycobacteria. Mice at particular stages of infection with *Salmonella typhi* or *Brucella abortus* can eliminate *Listeria monocytogenes* more easily from their tissues than can normal mice (1, 11). In the present studies vaccinia challenge of mice having a delayed-type sensitivity to *S. aureus* and elicited with specific antigen also resulted in tail swelling which was significantly less than normal mice, normal mice given staph antigen (SPL), and sensitized mice not given SPL (Fig. 2). Infectivity titrations also indicate that there is more than a log less of infectious virus in the
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Fig. 1. Changes in tail diameter of normal and *Mycobacterium tuberculosis* (TB)-sensitized mice after vaccinia challenge. The line for each group represents the average daily change in tail swelling for about 50 mice.

**Table 1. Infectivity titrations: tail tissue at peak day of swelling**

<table>
<thead>
<tr>
<th>Group</th>
<th>Pock formed/ml</th>
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<tbody>
<tr>
<td></td>
<td>Expt I</td>
</tr>
<tr>
<td>Normal</td>
<td>5.6 x 10^6</td>
</tr>
<tr>
<td>Normal + OT</td>
<td>1.3 x 10^6</td>
</tr>
<tr>
<td>TB sensitized</td>
<td>2.5 x 10^6</td>
</tr>
<tr>
<td>TB sensitized + OT</td>
<td>3.6 x 10^6</td>
</tr>
</tbody>
</table>

* At the peak day of swelling, tails were removed from five mice of each group and homogenized in saline containing antibiotics. Appropriate X2 dilutions were used for pock counts.

* Statistical analysis: students t test for difference of means of the five experiments.

* Normal group is not significantly different from normal + OT group.

* *Mycobacterium tuberculosis* (TB)-sensitized group is not significantly different from the normal + OT group.

* TB-sensitized group is significantly (P < 0.01) from normal and normal + OT groups.

When this work was initiated, experiments were performed to determine the effects of varying amounts of virus on this host; two other interesting differences between sensitized and control mice were observed after vaccinia challenge.

After intravenous injection of unpurified, unconcentrated virus, the early death observed by others using purified, concentrated virus (28) was not found in our mice; however, our test system was similar in that mice did die 5 to 7 days after being given 10^7 infectious units or showed severe tail swelling and damage along with swollen hind limbs when given about 10^6 infectious units. Less tail swelling with varying numbers of lesions was seen when 10^4 to 10^5 in-
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FIG. 2. Changes in tail diameter of normal and staphylococcus-sensitized mice after vaccinia challenge. The line for each group represents the average daily change in tail swelling for 12 to 15 mice.

TABLE 2. Deaths after vaccinia challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Deaths at day after challenge</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>20</td>
<td>0 7 6 1 0 0 0 0 0</td>
<td>70</td>
</tr>
<tr>
<td>Normal + OT</td>
<td>20</td>
<td>0 6 7 1 0 0 0 0 0</td>
<td>70</td>
</tr>
<tr>
<td>TB sensitized</td>
<td>13</td>
<td>0 2 1 1 0 0 0 0 0</td>
<td>31</td>
</tr>
<tr>
<td>TB sensitized + OT</td>
<td>14</td>
<td>0 0 0 0 0 0 0 0 0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mice were given $10^{7.3}$ infectious units of vaccinia intravenously; deaths were recorded over a 2-week period.

DISCUSSION

These experimental results demonstrate that mice with strong delayed-type allergy due to infection with mycobacteria or staphylococci are, after specific elicitation, more resistant to the various effects of vaccinia virus than control mice. The basic mechanism is not entirely clear as yet and is the subject of continued investigation.

The greatest enhancement of resistance to vaccinia occurs under rather exacting conditions of induction of strong cell-mediated sensitivity by infection accompanied by a critical amount of specific elicitation. These conditions for increase of nonspecific resistance with respect to vaccinia are in accord with those reported with respect to unrelated bacterial agents. The present experiments show that infection with H37Ra alone produces some re-
duction in both the amount of infectious virus found and in the amount of tail swelling produced. A similar result has been reported for ectromelia virus (6). However, the present experiments extend the investigation to the effects of elicitation on a virus infection, showing that a small amount of OT further decreases both the amount of virus and its effects. Exposure of immunologically committed lymphocytes to specific antigen is known to induce the liberation of a number of soluble products, including interferon, which are capable of influencing macrophages in a variety of ways (3). Investigation of the role of such products under the conditions of these experiments is not complete; however, positive evidence of interferon liberation under the conditions of these experiments has not been found (18). Conceivably, local production of interferon or other humoral factors might be involved; this is presently under investigation.

Infectivity titrations suggest that vaccinia is immediately associated with and replicates in cells of the tail tissue, which is a primary rather than a secondary site of replication. The particular cell involved has not been determined; but regardless of type, it would follow that conditions increasing phagocytes with enhanced digestive capacity, since these cells normally digest vaccinia readily (13), would result in a reduction in the amount of virus which, in turn, would result in less tail swelling and damage. Infectivity titrations suggest the idea that the observed increase in resistance to vaccinia may be associated with increased digestion of the virus, resulting in lower final titers of virus in tail tissue. This, rather than any loss of ability on the part of the host to respond to vaccinia with a hypersensitivity reaction, would seem to explain the significantly lower virus titers and tail damage in groups of sensitized mice given specific antigen when compared with control groups. It is hoped that comparative in vitro studies of the interaction of cells from similar groups of mice with vaccinia will answer the question more completely.

In these experiments, allergy due to infection with staphylococci differs from that induced by infection with mycobacteria in that infection alone has no inhibitory effect on either the amount of virus or its effects on mouse tails. In line with this, but using another system, Lenhart and Mudd (8) report that macrophages from staphylococcus-infected rabbits do not exhibit increased phagocytic and digestive abilities until reexposure to specific antigen. In view of the differences between these two bacteria it would seem that the known intracellular persistence of mycobacteria in host tissues might sustain elicitation whereas the relatively rapid fall in numbers of viable staphylococci (essentially an extracellular organism) in the mouse (21) would result in little or no continuing elicitation due to infection. Intracellular Toxoplasma gondii produce chronic infection in the mouse and behave like mycobacteria in that resistance is conferred against a variety of unrelated microorganisms and transplantable tumors (J. B. Hibbs, L. H. Lambert, J. S. Remington, Abstr. Reticuloendothel. Soc., p. 16, 1971); in the case of Toxoplasma it is difficult to make an estimation of the role of elicitation.

Perhaps chronic infection which induces delayed-type allergy and at the same time continuously produces small amounts of specific antigen capable of evoking increased activities of phagocytic cells is the basis of what has become known as "infection-immunity"—the situation observed by many in syphilis, tuberculosis, chlamydia-induced disease, and other infections (20, 25, 26) in which the host is highly resistant to reinfection under certain conditions of persistence of the initial infection. In fact it would seem that experimental evidence continues to accumulate supporting Metchnikoff's original observations and ideas that "The acquisition of immunity against microorganisms is, therefore, due not only to positive and negative chemotaxis, but also to the perfection of phagocytic and digestive powers of leucocytes—a general superactivity and adaptation of the phagocytic reaction of the immunized animal is produced" (12). Though it is becoming more and more evident that cell-mediated phenomena play as great a role in host resistance as specific humoral antibody in the final handling of human virus infection (5, 7, 24), it is also becoming evident that there are both specific and nonspecific cellular mechanisms which may affect both intra- and extracellular organisms as well as tumors (27; Hibbs, Lambert, and Remington, Abstr. Reticuloendothel Soc., p. 16, 1971).

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LITERATURE CITED


