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Immune Mechanism in *Pasteurella multocida*-Infected Mice

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Various immunizing procedures were tested for their effect on the growth of *Pasteurella multocida* in specific pathogen-free CD-1 mice. Two injections with killed vaccine incorporated into Freund complete adjuvant gave excellent protection against parenteral challenge, but did less to control the growth of an aerogenic inoculum. Protection with adjuvant-treated preparations was always superior to that seen with non-adjuvant-treated vaccines. A killed saline-suspended vaccine introduced aerogenically marginally increased resistance to small (2 to 5 mean lethal doses) aerogenic challenges. Live *Mycobacterium bovis* (BCG) vaccine immunopotentiated a single dose of heat-killed *P. multocida* vaccine in terms of the protective immune response against a subsequent footpad challenge. Hyperimmune mouse serum, administered intraperitoneally, intramuscularly, or intravenously 1 to 7 days prior to subcutaneous challenge with 500 to 5,000 *P. multocida,* was highly protective. Mice injected in the thigh with 0.2 ml of hyperimmune serum prior to footpad challenge frequently developed a severe inflammatory response with local swelling within 48 h; these lesions often became severely abscessed with time. The passively protected, footpad-infected mice developed active immunity against a subsequent challenge given into the opposite footpad.

Although immunity to *Pasteurella multocida* infections can be demonstrated in appropriately immunized animals (2, 3), there are still many unanswered questions regarding the mechanism(s) involved. Studies of the antigenic structure of *P. multocida* substrains have resulted in the development of successful commercial vaccines against a variety of economically important diseases in birds and cattle (3). Passive transfer studies indicate that this immunity is largely humorally mediated (2), but the precise mechanism of this protection is unclear (5), largely because most protection studies have relied on mortality/survival data alone. However significant these differences in survival rates may be (2), such studies tell little about the means by which the vaccinated host controls the growth of the parasite in vivo, so that the actual mechanism of bacterial inactivation remains something of an enigma. Detailed growth curves obtained using both actively and passively immunized animals have revealed the nature of the immune response against a number of microbial parasites (6), and there seems little reason to doubt that a similar approach will be just as fruitful for *P. multocida.* With this in mind, the growth of virulent *P. multocida* in both actively and passively immunized mice was compared quantitatively in the hope that the manner by which the immune host was able to eliminate this extraordinarily virulent pathogen from the tissues could be determined.

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MATERIALS AND METHODS

Animals. Specific pathogen-free male and female CD-1 mice (Charles River Farms, Wilmington, Mass.), weighing an average of 25 g, were used throughout. They were maintained on sterile bedding and were fed sterile food and water ad lib.

Organisms. *P. multocida* 5A was originally isolated from turkeys. Details of laboratory maintenance and growth characteristics have been described previously (5). This strain has a mouse mean lethal dose (LD_{50}) of 1 viable organism when introduced by the intravenous, intraperitoneal, subcutaneous (footpad), or intradermal routes and about 10 organisms when introduced by the aerogenic route.

Mycobacterium bovis BCG Pasteur (TMC 1011) was obtained from Trudeau Mycobacterial Culture Bank, Saranac Lake, N.Y. The organisms were kept as a frozen suspension (−70 C), thawed, and homogenized before injection as described previously (7). Viable counts of suitable 10-fold dilutions were carried out on Middlebrook 7H10 medium. Plates were incubated at 37 C for 3 weeks in sealed plastic bags.

Vaccines. *P. multocida* was grown in shaken heart infusion broth (Difco, Detroit) and heat-killed as described previously (5); the cells were centrifuged and resuspended in 1/10 of the original volume

of saline and freeze-dried. Most of the mice were injected with 200 μg of heat-killed *P. multocida* suspended in 0.2 ml of saline or in Freund complete adjuvant (FCA) (4). Mice were injected subcutaneously in multiple sites across the back and abdomen (a total volume of 0.2 ml), and a repeat dose was usually given 7 days later. In one experiment, the mice were immunized with an aerosol generated from a suspension of 5 mg of heat-killed *P. multocida* per ml of saline for 30 min in a Middlebrook chamber (Tri-R Instruments, Rockville Center, N.Y.). A similar dose of aerogenic vaccine was given 7 days later.

Corynebacterium parvum vaccine (7 mg [dry weight] of cells per ml) was obtained from Wellcome Laboratories, Triangle Park, N.J., and 0.05 ml containing 200 μg of *C. parvum* with 200 μg of killed *P. multocida* was injected into a hind footpad 4 to 7 days prior to challenge. Some mice received 0.1 ml of *C. parvum* vaccine intravenously (9) with 200 μg of *P. multocida* injected into one footpad 4 days later. These mice were then challenged in 4 or 8 days.

Challenge methods and bacterial enumeration in vivo. Details of procedures for intravenous, subcutaneous (footpad), and aerogenic challenge with live *P. multocida* have been described earlier (5). Bacterial counts carried out on homogenates of selected tissues taken from five randomly selected mice per time point were performed with previously published techniques (7). Where counts were made on the footpad, the whole foot was removed and rinsed in 70% alcohol, and the flesh was stripped from the bones with a Virtis high-speed blender (8). All dilutions were carried out in Hanks balanced salt solution containing 1% fetal calf serum and plated on heart infusion agar incubated at 37 C for 48 h.

Passive protection tests. Hyperimmune serum was obtained from mice repeatedly vaccinated with 10^8 heat-killed *P. multocida*. The mice resisted an intravenous challenge with 10^4 viable *P. multocida* (5). They were then bled by heart puncture and the serum was stored without preservative at −20 C. Undiluted serum was injected intravenously, intraperitoneally, or intramuscularly (thigh) in volumes of 0.1 to 0.2 ml 24 h prior to challenge. The challenge inoculum (500 LD_{50}) was given either intraperitoneally or subcutaneously (via a footpad). Control mice received 0.2 ml of normal mouse serum.

RESULTS

Intravenous challenge of vaccinated mice. The growth of *P. multocida* was followed in the tissues of animals receiving two weekly injections of killed *P. multocida* vaccine, with and without adjuvant, prior to challenge. The mice were challenged by the intravenous route with 10^3 viable organisms given 7 days after the second antigen dose (Fig. 1). The growth pattern was much the same whether the vaccine was incorporated into FCA or Freund incomplete adjuvant (FIA). In all of the vaccinated mice, the liver and spleen counts obtained 5 h after challenge were 100-fold lower than in the normal controls. However, only those animals

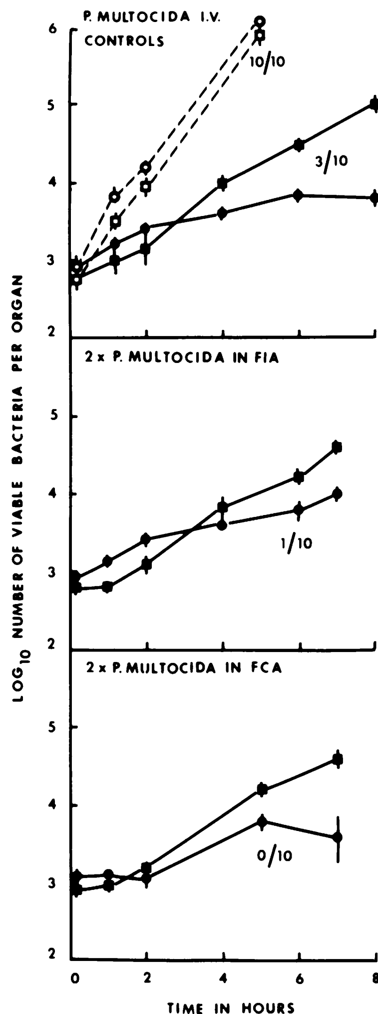

FIG. 1. *Growth of an intravenous P. multocida challenge (10^3 viable organisms) in the livers (circles) and spleens (squares) of mice vaccinated subcutaneously with two weekly doses of heat-killed P. multocida suspended in saline (top), FIA (middle), or FCA (bottom). The normal control curve is represented by the broken lines. The vertical lines represent the standard error of the mean of five determinations. The figures refer to the number of deaths/number of mice in each challenged group.*

receiving the vaccine in FCA seemed to be fully protected against the lethal effects of the challenge infection.

Aerogenic challenge. Earlier work has suggested that protection may be difficult to establish in the mouse against aerogenically introduced parasites (5, 15). *P. multocida* induces a more slowly developing systemic infection after aerogenic inoculation of normal mice with moderate numbers of viable bacilli. However, both

normal mice, as well as most of those receiving vaccines without adjuvant were dead by 72 h (Fig. 2). Suspension of the vaccine in FCA resulted in a slower spread to the spleen by the population growing rapidly in the lung (18 h compared with 7 h in the controls). As a result, heavy growth was not observed on many of the liver and spleen plate counts in this group (Fig. 2). Many of the mice receiving the vaccine with adjuvant managed to survive the infection despite extensive growth by the lung population. The maximum lung counts for the mice receiving the *Pasteurella* vaccine in FIA was only about 1/10 of the control counts, whereas those receiving the FCA preparation were as much as

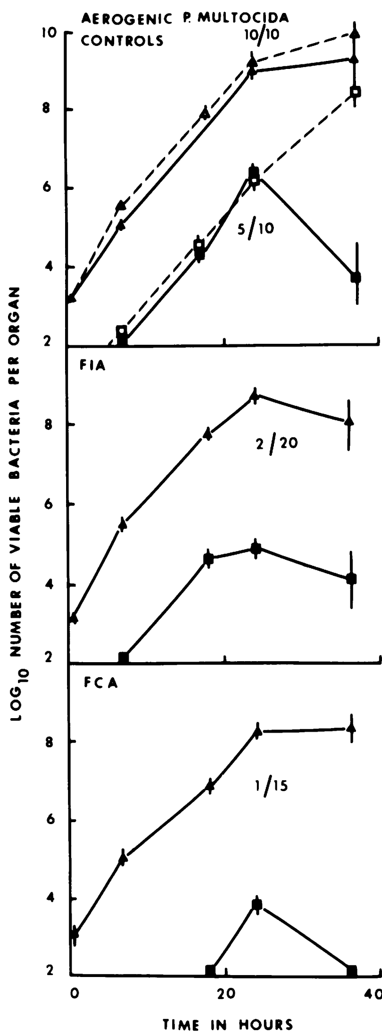

FIG. 2. *Growth of an aerogenic challenge dose of P. multocida in mice vaccinated with two doses of heat-killed P. multocida vaccine. See legend to Fig. 1 for further details.*

100-fold lower. By 36 h, all of the unvaccinated control mice showed very high bacterial counts in the lungs ($10^{10.0}$) and spleens ($10^{8.5}$), and by 48 h these mice were dead. On the other hand, the viable counts for many of the vaccinated mice were already beginning to decline by this time and only 10 to 15% of the adjuvant-treated mice succumbed to the infection.

The resistance of mice immunized aerogenically with a killed *P. multocida* vaccine was variable even when a small aerogenic challenge ($10^{1.8}$ viable organisms) was used. Survival rates of 10/15 (66%) for the vaccinated mice compared with 6/15 (40%) for the untreated controls were typical and were not significantly different from one another. However, 24 h after infection, bacterial counts carried out on the lungs, livers, and spleens of the vaccinated group were 10 to 20 times lower than in the control animals, which was statistically significant, although it was clearly not enough to change the overall survival rate. When the aerogenic challenge dose was increased 10-fold, no evidence of protection could be detected.

Subcutaneous challenge. Multiple doses of heat-killed *P. multocida* vaccine induce an effective resistance against 10 to 100 LD_{50} challenge doses given by the intravenous, intraperitoneal, or subcutaneous routes (5). A single dose of vaccine had little protective value (18/20 deaths within 24 h of being infected with 500 live *P. multocida*). Attempts were made to immunopotentiate the response by first stimulating the draining lymph node with live BCG (13). BCG Pasteur (2×10^5 viable units) were injected into one hind footpad, followed 12 days later by a single injection of 0.2 to 2.0 mg of heat-killed *P. multocida* suspended in saline. Mice challenged with 5×10^3 to 15×10^3 *P. multocida* 8 to 12 days later via the same footpad showed considerable protection (Table 1). When this experiment was repeated using a higher dose of BCG (1.5×10^6 viable organisms), even better protection was observed (Fig. 3). No protection was observed when the live BCG vaccine was administered alone 21 days before challenge.

Increased protection also occurred when 200 μg of heat-killed *C. parvum* vaccine was mixed with 200 μg of heat-killed *P. multocida* and injected into a hind footpad. The mice were challenged 4 days later with 5,000 viable *P. multocida* injected into the same footpad (Fig. 4). Reduced protection was observed if the challenge was made 14 days after vaccination or if the *C. parvum* was given separately (intravenously) from the heat-killed *P. multocida* (subcutaneously).

Passive protection tests using hyperim-

TABLE 1. *Protection of mice against subcutaneous (footpad) P. multocida challenge after previous injection with viable BCG and heat-killed P. multocida*

Immunization schedule		Dead/total[b] after challenge on:		
Day −14	Day 0	Day 8	Day 10	Day 12
BCG[a]	0.02 mg of heat-killed *P. multocida*	5/5	5/5	4/5
BCG	0.2 mg of heat-killed *P. multocida*	5/5	3/5	2/5
BCG	2 mg of heat-killed *P. multocida*	2/5	1/5	1/5
BCG	Nil	4/5		
Nil	0.2 mg of heat-killed *P. multocida*	5/5	5/5	5/5
Nil	Nil	5/5		

[a] Animals were given 2×10^5 *M. bovis* BCG Pasteur into the right hind footpad. Dead *P. multocida* were injected into the same footpad 14 days later, and challenged with 5×10^3 live *P. multocida* 8 to 12 days later.

[b] Deaths recorded for 7 days.

mune mouse serum. Protection against an intraperitoneal challenge with 1,000 viable *P. multocida* was observed when the mice were first injected with 0.2 to 0.4 ml of hyperimmune anti-*P. multocida* mouse serum. Less protection occurred if the hyperimmune serum and the challenge population were injected separately, at the same time. For instance, when a subcutaneous (footpad) challenge was given, only 6/10 mice survived when given hyperimmune serum intravenously (Fig. 5). Injection of the immune serum into the footpad was less effective than the intraperitoneal or intravenous routes, presumably because of the small volume of serum (0.04 ml) that could be successfully introduced into the footpad. When the 0.2 ml of hyperimmune serum was injected into the thigh 24 h prior to a footpad challenge with 500 LD_{50} doses of viable *P. multocida*; 26/30 of the mice survived (Fig. 6). Removal of the draining popliteal lymph node 7 days prior to serum treatment did not reduce this protection. Five out of ten challenged animals were even protected when the interval between the administration of the immune serum and the challenge was increased to 7 days. Little or no protection was observed, however, if the serum was injected into the leg immediately after the footpad challenge. Control animals receiving 0.2 ml of normal mouse serum were not protected.

Some of the survivors from these serum protection studies developed severely swollen feet, usually beginning 2 to 3 days after the challenge (Fig. 7). Eventually, many of these feet developed multiple abscesses (Fig. 8A) exhibiting a predominantly polymorphonuclear leukocyte response (Fig. 8B), which persisted for at least 14 days. A purulent exudate could be expressed from these abscesses, with bacterial counts of up to 10^8 viable *P. multocida* per ml of pus. Injection of organisms recovered from the pus into normal mice revealed that the bacilli within the abscess were still fully mouse virulent, indicating that the long-term survival of the serum-treated mouse was due to de novo acquired resistance induced by the local *Pasteurella* infection and not to the attenuation of the *P. multocida* within the footpad. Injection of 10^5 viable *P. multocida* into the contralateral footpad in mice that had developed severely swollen feet 14 or more days previously did not result in the death of any of the doubly challenged mice.

DISCUSSION

The efficacy of the heat-killed *Pasteurella* vaccine was considerable provided that multiple doses of immunogen were used (5). However, even under these circumstances, reduced protection was usually observed when an aerogenic challenge was employed. Even when the *Pasteurella* vaccine was suspended in FCA, the severity of the aerogenic infection was only mitigated, never prevented (Fig. 2). Introduction of vaccine directly into the lung should stimulate the production of specific secretory immunoglobulin A antibodies, analogous to that recently demonstrated for a *Pseudomonas aeruginosa* rabbit infection model (14). Such development of local antibody production should enhance the ability of the host to limit the growth of this parasite when it too is introduced directly into the lung. However, protection only occurred when very modest challenge doses were used, and the respiratory challenge remains the most stringent test of the protective value of any *Pasteurella* vaccine, and should certainly be used wherever a respiratory mode of spread appears to occur during the natural course of the disease (2, 3, 10). It is difficult to believe that an organism as virulent as *P. multocida* can remain within the nasopharynx (far less in the lungs) of unprotected individuals for any length of time without the development of clinical disease. Presumably, the normal healthy lung is somehow able to eliminate small numbers of bacilli as fast as they reach the alveoli (12), a situation perhaps analogous to that occurring in the draining lymph nodes of serum-protected mice (Fig. 6). Interestingly, unlike the situation reported for turkeys (1), normal mice housed with aerogenically (or intravenously) infected animals (all of which died as a result of an overwhelming systemic infection) showed no evidence of pneu-

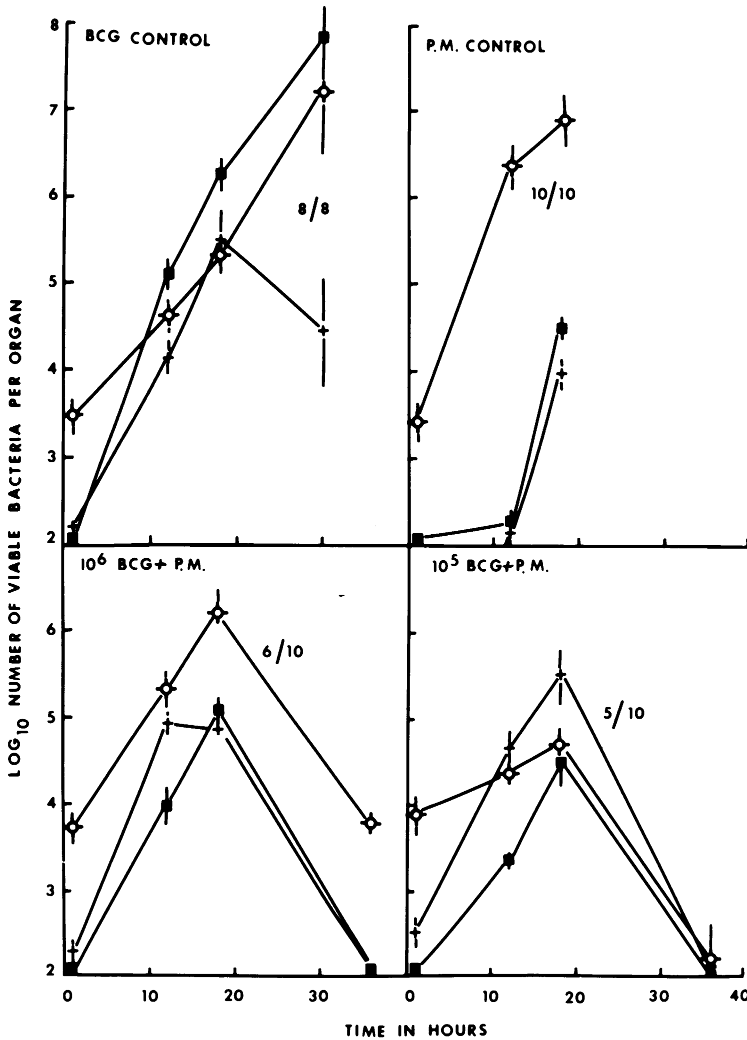

FIG. 3. *Growth of 5,000 viable P. multocida in the footpads (circle), spleens (square), and popliteal lymph nodes (crosses) of mice vaccinated 16 days previously with 10^5 (right bottom) or 10^6 viable BCG Pasteur into the footpad and then 4 days previously with 200 μg of heat-killed P. multocida into the same footpad. The appropriate controls are shown in the top sections.*

monic spread by the disease to the newly added animals.

Serum protection requires the injection of relatively large doses (0.2 to 0.4 ml) of undiluted hyperimmune mouse serum, introduced either intramuscularly or intraperitoneally at least 24 h prior to challenge. Protection correlated with a prevention of hematogenous spread by the organisms from the footpad to the liver and spleen. However, passive serum protection could also be achieved against an intravenous challenge in which a substantial proportion of the inoculum reached the liver and spleen within minutes as the organisms were rapidly cleared from the blood. Serum treatment resulted in the absence of soaring numbers of bacteria in the blood, a condition which normally develops early in the infection; growth in the liver and spleen was significantly reduced but never prevented. However, by 24 h the systemic infection was invariably eliminated and most of the serum-treated mice survived.

Introduction of hyperimmune serum into the leg immediately below the popliteal lymph node effectively prevented the spread of the footpad challenge into the bloodstream, and virtually all of the animals survived despite the development of local abscesses at the site of

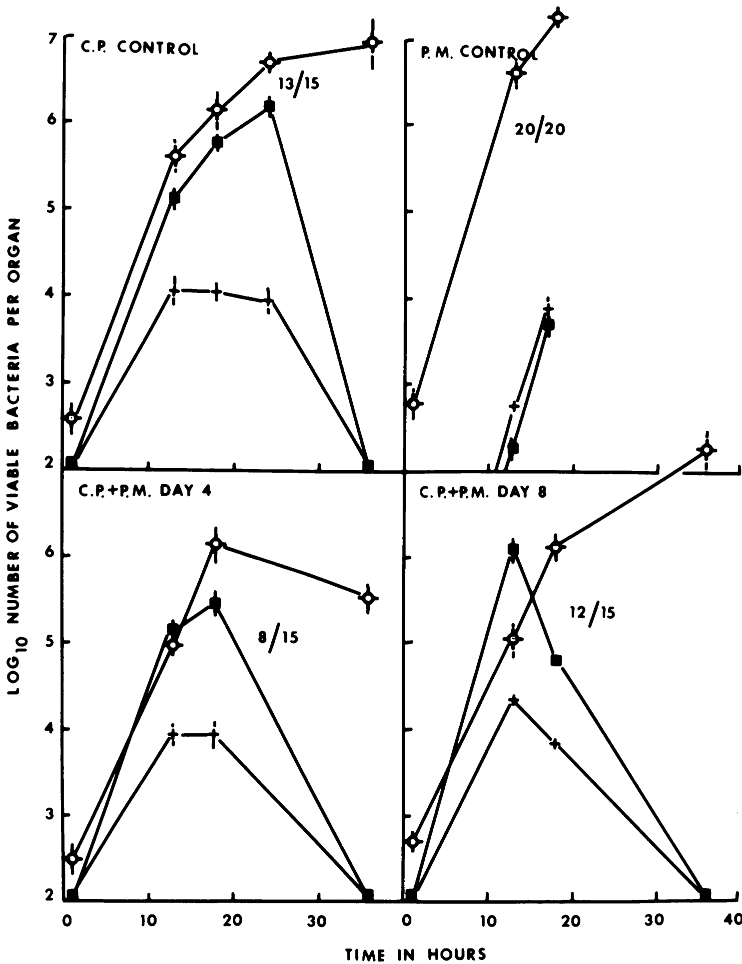

FIG. 4. *Growth of 400 viable P. multocida in the footpads of mice vaccinated 4 days previously (left bottom) or 8 days previously (right bottom) with a mixture of 350 μg of heat-killed C. parvum and 200 μg of heat-killed P. multocida injected into the footpad. Mice receiving C. parvum or P. multocida alone are shown in the top section. Circle, footpad; square, spleen; cross, popliteal lymph node.*

inoculation. The passive immune response was extraordinarily effective, so that the mice lived despite viable *P. multocida* counts within the swollen footpads equal to those found in the livers and spleens of moribund control mice (Fig. 5). An explanation for this intriguing paradox is not presently obvious.

Some of the animals given hyperimmune serum did not develop swollen feet after challenge, and in fact no viable bacilli could be detected in the footpads, popliteal lymph nodes, spleen, or blood 24 h later. This resistance to challenge slowly declined so that by 21 days none of these animals reinfected with 10^4 viable *P. multocida* survived, regardless of whether the second challenge was introduced into the original or the contralateral footpad. This difference was explained on the basis of a complete inactivation of all of the challenge organisms at the inoculation site and within the draining lymph node so that none of the bacilli reached the bloodstream. As a result, there was not a sufficient antigenic stimulus to the reticuloendothelial system to induce a significant active immunity. On the other hand, when the infection established itself within the footpads of the serum-treated mice, the viable organisms were effectively prevented from passing in to the bloodstream, but sufficient antigenic material drained from the footpad abscesses to induce a long-lasting active immunity.

The histopathology of these footpad lesions

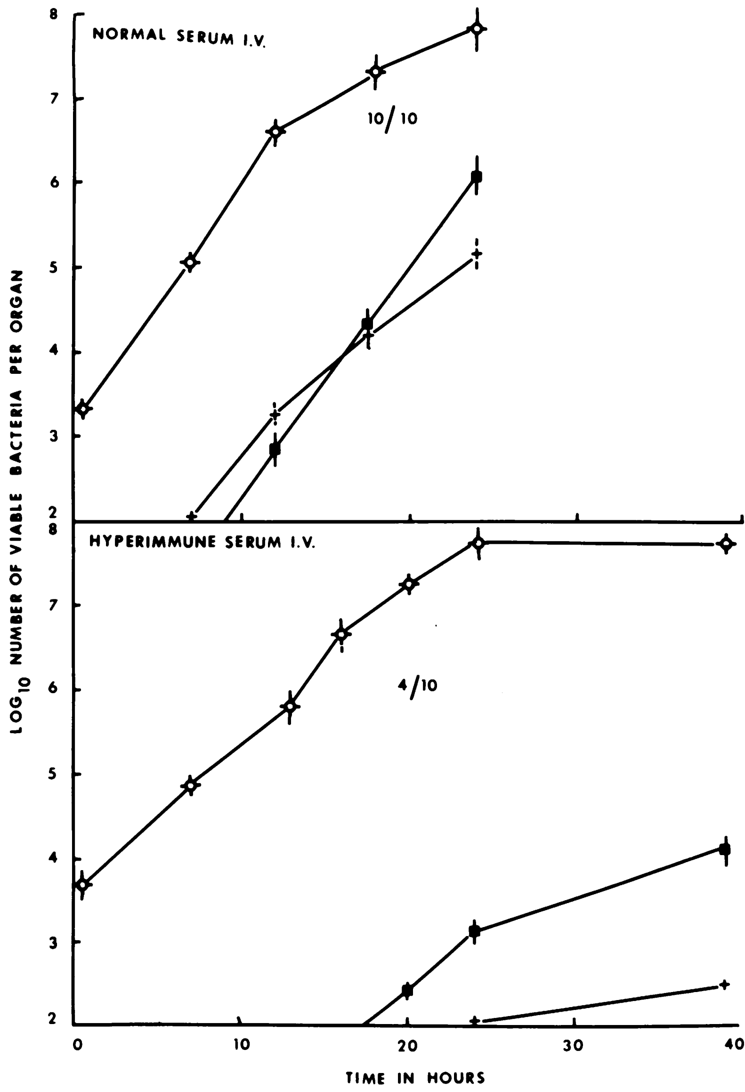

FIG. 5. *Growth of P. multocida in the footpads of mice injected intravenously with 0.2 ml of hyperimmune mouse serum 24 h previously. The normal serum controls are shown in the top section.*

offers some insight into the nature of the immune response to this organism. The purulent abscesses contained enormous numbers of polymorphonuclear leukocytes, with very few mononuclear cells (Fig. 8B). Interestingly, human infections (often associated with dog or cat bites) are also septic in nature (11). Thus, the mouse footpad lesion may provide a valid experimental model for assessing chemotherapeutic and immunotherapeutic measures for use in such human infections. On the other hand, bovine and porcine infections primarily involve the lung, presumably following the transport of virulent *P. multocida* from the nasopharynx down the bronchial tree to the alveolar spaces (2). Studies of the progress of experimental infections in large animals are both difficult and expensive, so the aerogenic mouse model may be valuable as a means for studying those host changes associated with the containment of naturally acquired pasteurellosis in vaccinated cattle. As long as the precise mechanism by which the effectively immunized host expresses this resistance at a cellular and a tissue level remains unknown, the development of more effective immunogens and immunizing regimens must remain largely empirical, despite the considerable economic importance of these animal infections. Clearly, a great deal more study will be required before we fully understand this fascinating disease.

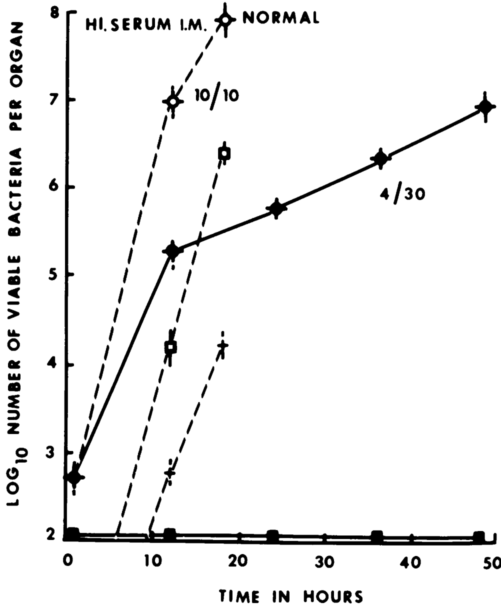

FIG. 6. *Growth of P. multocida in the footpads of mice injected intramuscularly with 0.2 ml of hyperimmune serum into the thigh 24 h previously. The broken lines represent the growth curves for normal mice.*

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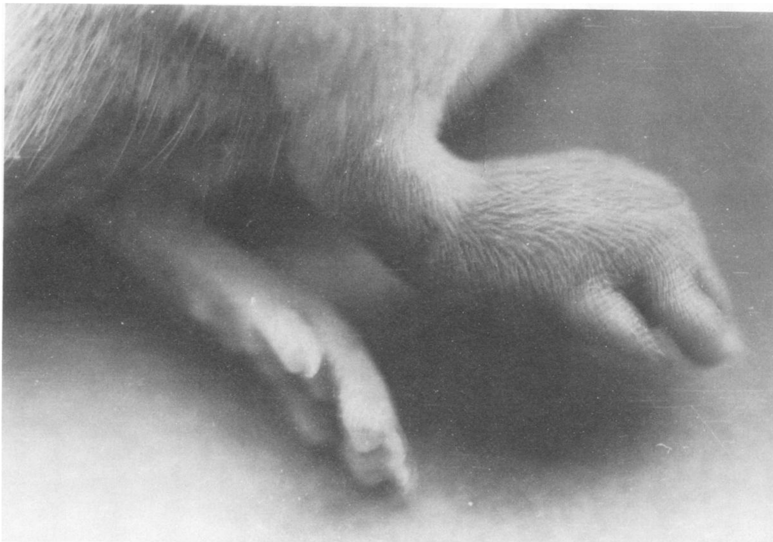

FIG. 7. *Swollen foot at day 6 in mouse given 0.2 ml of hyperimmune serum intramuscularly (thigh) 24 h before subcutaneous (footpad) challenge with 500 viable P. multocida.*

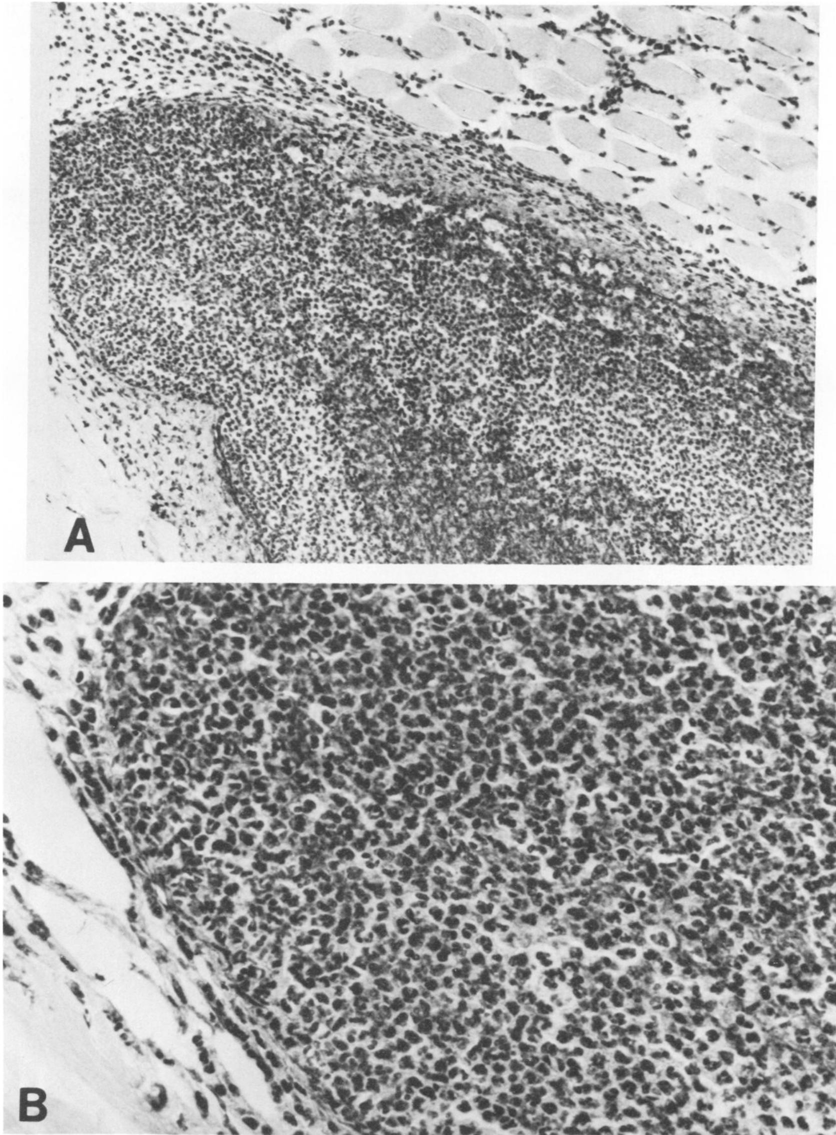

FIG. 8. *(A) Lesion in foot of mouse shown in Fig. 7 (hematoxylin and eosin, ×150). (B) Infiltrate is almost entirely polymorphonuclear, as shown in this enlargement (×400).*

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