MECHANISM OF IMMUNITY TO *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* (BUCHANAN, 1911) IN MICE USING INACTIVATED VACCINE

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ABSTRACT

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Mice can be passively protected against infection with *Corynebacterium pseudotuberculosis* with hyperimmune rabbit serum prepared against whole bacterial cells. The mechanism of immunity depends on the inhibition of secondary multiplication of the bacteria, not on antitoxic activity or enhanced phagocytosis and destruction.

Passive transfer of macrophages obtained from mice immunized with inactivated vaccine to susceptible mice which were subsequently challenged showed that the macrophages have no specific immunizing properties.

INTRODUCTION

In previous publications it was reported that mice can be adequately protected against subacute infection with *Corynebacterium pseudotuberculosis* (Buchanan, 1911) when they are immunized with whole bacteria alone, and it has also been shown that the antigen which is responsible for inducing the protective immunity is located primarily in the cell wall (Cameron & Minnaar, 1969; Cameron, Minnaar & Purdom, 1969).

During routine testing of vaccine it was, however, found that an absolute immunity was not always obtained. Although the death rate was markedly reduced, occasionally immunized mice eventually succumbed to infection if they were observed for a period of 3 weeks or longer. It seemed as though the bacteria used for challenge were usually destroyed completely, but in rare instances a few organisms survived and were able to multiply and eventually cause a lethal infection. It is common knowledge that in certain bacterial diseases the defence mechanisms are essentially humoral; in some, specific levels of antitoxin will afford total protection while in others opsonization and effective phagocytosis is critical for an effective immunity. In other bacterial diseases such as tuberculosis, listeriosis, brucellosis and tularaemia, cellular immunity is of prime importance. In these instances enhanced reactivity of the phagocytic cells, particularly the macrophages, is essential in order to afford a solid immunity (Mackaness & Blanden, 1967). A noteworthy characteristic of the diseases mentioned above is that the causative organisms are all intracellular parasites.

It must also be realized that inactivated vaccines are of doubtful value as immunizing agents against these infections; significant immunity can only be obtained if live vaccines are used.

C. pseudotuberculosis is also a facultative intracellular parasite and, in view of the facts mentioned above, cellular immunity may be necessary in order to afford solid protection. Experiments were therefore conducted to discover whether humoral or cellular immunity is primarily responsible for protection and to follow the changes in the bacterial population after infection of immunized and non-immunized mice.

MATERIALS AND METHODS

Strain of bacteria

C. pseudotuberculosis strain 137B was originally obtained from Prof. H. R. Carne* and was used for preparation of vaccine as well as for challenge of experimental mice.

Preparation of vaccine

Aluminium phosphate adjuvant vaccine suitable for administration to sheep was prepared as described previously (Cameron & Minnaar, 1969). It contained a final concentration of either 0,5 or 1,25 per cent packed cells. This is equivalent to about 0,1 or 0,25 mg per 0,2 ml mouse dose respectively.

For the preparation of oil adjuvant vaccine, 50 mg of washed dry bacteria were suspended in 5,0 ml of 0,85 per cent NaCl containing 0,5 per cent formalin. After standing at room temperature overnight, the suspension was emulsified with 5,0 ml Freund's complete adjuvant (Difco)**. The final vaccine thus contained 0,25 mg dry bacteria per 0,1 ml dose.

Vaccine used for the production of hyperimmune whole cell rabbit antiserum (HRA) was prepared from bacteria grown on blood tryptose agar plates. The organisms were collected by means of a curved glass rod and suspended in 0,85 per cent NaCl containing 0,5 per cent formalin. The concentration of bacteria was adjusted to give a final density of 1,5 per cent packed cells. This concentration is equivalent to approximately 5×10^8 to 10^9 bacteria per ml or 2,0 mg dry bacteria per ml. After standing at room temperature overnight, a portion of the suspension was emulsified with Freund's complete adjuvant.

Production of exotoxin and antitoxin

Potent exotoxin was produced in static cultures incubated for 48h. Details of the method will be given in a forthcoming publication. The same antitoxin was used as that employed previously (Cameron & Smit, 1970).

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Immunization of mice

Groups of 20 6-week-old female albino mice were used in all the experiments. Aluminium phosphate adjuvant vaccine was administered subcutaneously, each mouse receiving two injections each of 0,2 ml, at an interval of 4 weeks. Oil adjuvant vaccine was injected in doses of 0,1 ml using the same schedule. In both instances the dose was divided and given at two different sites. The mice were challenged 2 weeks after the second injection.

Preparation of hyperimmune whole cell antiserum (HRA)

For the preparation of HRA, six rabbits were immunized as follows:

Each rabbit was initially given 8,0 ml of bacterial suspension emulsified with Freund's complete adjuvant subcutaneously. The total quantity was divided into eight aliquots and injected at different sites along the neck and back. After 3 weeks rest, a series of intravenous injections of vaccine without adjuvant was given, as follows:

Day 1 — 0,1 ml
Day 2 — 0,3 ml
Day 3 — 0,6 ml
Day 8 — 0,6 ml
Day 9 — 0,6 ml
Day 10 — 0,8 ml
Day 15 — 1,0 ml
Day 16 — 1,0 ml
Day 17 — 1,0 ml

The rabbits were bled by cardiac puncture 14 days after the last injection and the serum stored at −20°C.

Toxin neutralization tests

The antitoxin potency of sera was assayed by determining its ability to neutralize the necrotizing effect of exotoxin on rabbit skin.

Five-fold serial dilutions of exotoxin were made in 0,85 per cent NaCl and 0,1 ml of each dilution was transferred to a clean tube, to which 0,1 ml of serum was added. After standing at room temperature for 30 min, 0,1 ml of each of the toxin-antitoxin mixtures was injected intradermally into rabbits. Care was taken to use skin areas which showed no active hair growth. The diameter of the reactions was measured 18 hours later and the surface area calculated.

Agglutination tests

Tube agglutination tests were done according to standard principles. Details of the procedure and preparation of antigen will be given in a forthcoming publication.

Passive protection tests

Groups of 20 12-week-old female albino mice were used in all the experiments. Antiserum or normal rabbit serum was administered either intravenously or intraperitoneally depending on the requirements of the experiment. The mice were challenged 18 to 24 hours later except where otherwise indicated.

Macrophage transfer

The procedure employed was based on the method of Miki & Mackaness (1964). Peritoneal macrophages were obtained from mice which had been immunized with oil adjuvant vaccine. Cells were collected essentially as described by Stuart (1967). The mice were killed with chloroform, the abdominal skin was removed and 2,5 ml Hank's solution containing 10 units heparin per ml were injected intraperitoneally. The abdomen was massaged gently and 1,0 to 1,5 ml fluid was aspirated. The macrophage suspensions from all the mice were pooled in a silicone coated Erlenmeyer flask and kept in an ice bath. The cells were deposited by centrifugation at 500 g for 5 to 10 min and resuspended in Hank's solution without heparin, using a volume of 0,5 ml for resuspending the cells collected from each group of four mice. The suspension thus obtained, contained approximately 8×10^8 macrophages per ml. Each recipient mouse was given 0,5 ml of this concentrated suspension intraperitoneally.

Macrophages obtained from normal mice were similarly collected and transferred to control recipients.

The mice were challenged 18 hours later by the intra-peritoneal injection of 2×10^6 live bacteria.

Groups of donor mice were challenged at the same time to prove that they were immune at the time that macrophages were collected.

Absorption of antisera

Antisera were absorbed with heat-killed bacteria as described by Ekstedt & Yoshida (1969).

Opsonization of bacteria

Two ml of a bacterial suspension containing 5×10^8 live bacteria per ml were centrifuged and resuspended in 2,0 ml undiluted hyperimmune rabbit serum. The suspension was shaken in a waterbath at 37°C for 30 min and the bacteria deposited by centrifugation. The sedimented bacteria were taken up in 20 ml 0,85 per cent NaCl and diluted further to give a final concentration of 5×10^6 bacteria per ml.

Challenge of experimental mice

The procedure for preparing shake cultures and enumerating bacteria in frozen stock challenge suspensions has been described previously (Cameron & Minnaar, 1969). The challenge suspensions were diluted to give the desired number of live bacteria in 0,2 ml. Mice were challenged with different doses of live bacteria either intravenously or intraperitoneally depending on the requirements of a particular experiment.

Enumeration of live bacteria in the peritoneal cavity

Mice were prepared as described for the collection of macrophages. Following the intraperitoneal injection of two ml 0,85 per cent NaCl approximately 1,0 ml was aspirated and deposited in cooled tubes. Tenfold serial dilutions were made using distilled water for the first dilution to facilitate lysis of the macrophages. The subsequent dilutions were made in 0,85 per cent NaCl and duplicate plate counts were prepared by spreading 0,1 ml of the 10^{-2} and 10^{-3} dilutions on blood tryptose agar plates. The colonies were counted after incubation at 37°C for 48 hours. Three mice per group were assayed at each time interval.

RESULTS

The potency of aluminium phosphate adjuvant vaccine, which is routinely prepared for administration to sheep, is tested in mice before it is issued. The vaccine contains a final concentration of 0,5 per cent packed cells; this is equivalent to approximately 0,1 mg dry bacteria per 0,2 ml, which is the standard dose used for immunizing mice. Although it has been shown that mice can be immunized with two doses of 0,04 mg dry bacteria (Cameron & Minnaar, 1969) it was found occasionally that mice which had received two injections of 0,1 mg each did not exhibit a solid immunity. A typical result of this kind is shown in Fig. 1. Despite

some immunity as indicated by the retarded death rate the majority of the immunized mice eventually died.

This phenomenon is much less evident when a higher dose of antigen is used. As shown in Fig. 2, mice which were immunized with two doses of 0,5 mg each were considerably more resistant to infection. A good immunity can therefore be obtained with an inactivated vaccine provided an adequate dose is used.
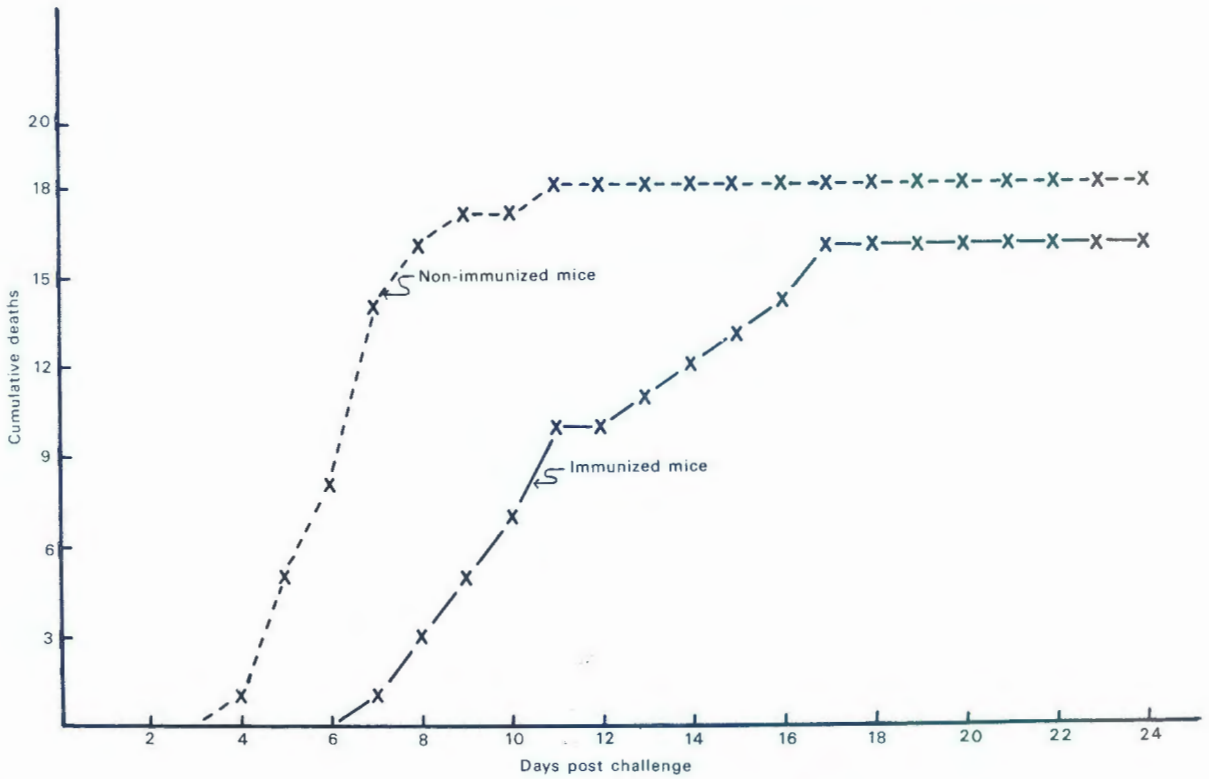

FIG. 1   Resistance of mice immunized with low doses of vaccine. Mice were immunized with two doses of 0,1 mg dry bacteria and challenged with 2×10^5 live bacteria intravenously.
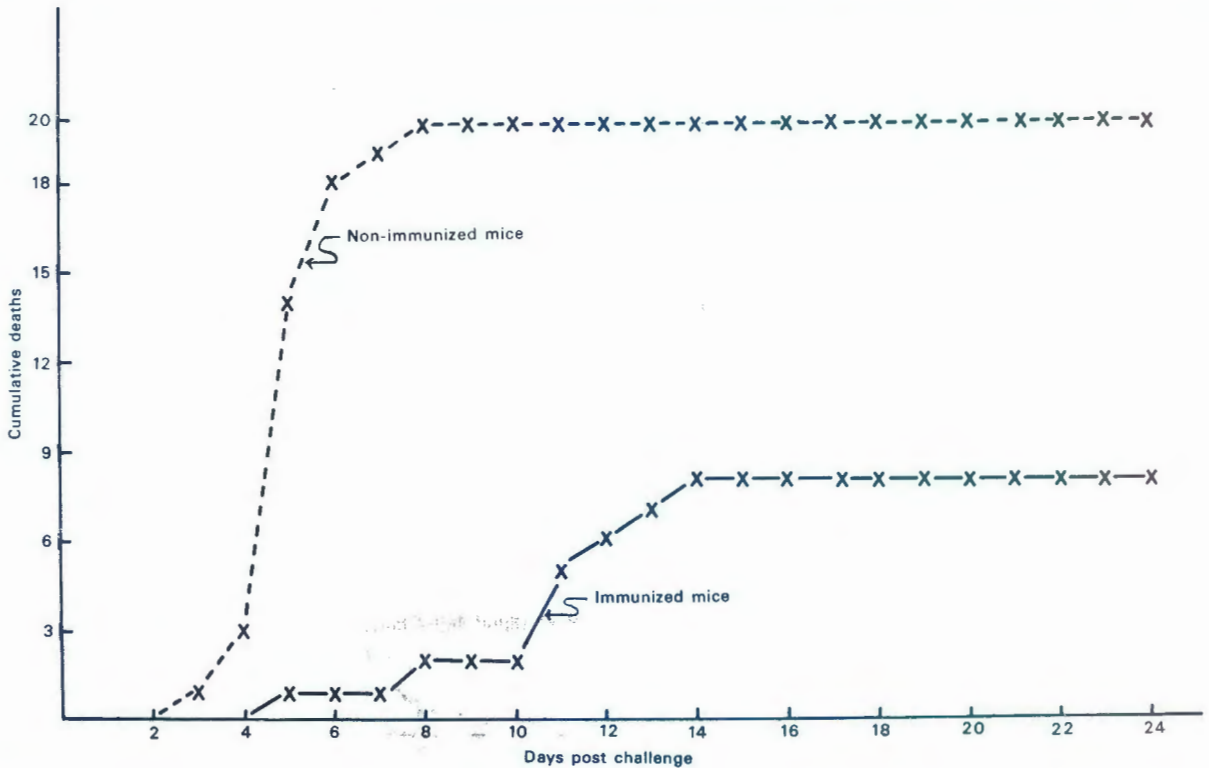
FIG. 2   Resistance of mice immunized with high doses of vaccine. Mice were immunized with two doses of 0,5 mg dry bacteria and challenged with 2×10^5 live bacteria intravenously.

It was nevertheless of interest to investigate the mechanisms of immunity in order to obtain a better understanding of the processes involved.

The results of an experiment in which the role of macrophages was investigated are given in Fig. 3.

Mice which received macrophages harvested from immunized animals were slightly more resistant to infection than non-immunized controls. Essentially the same was also found for mice which had received macrophages from normal mice. Compared with
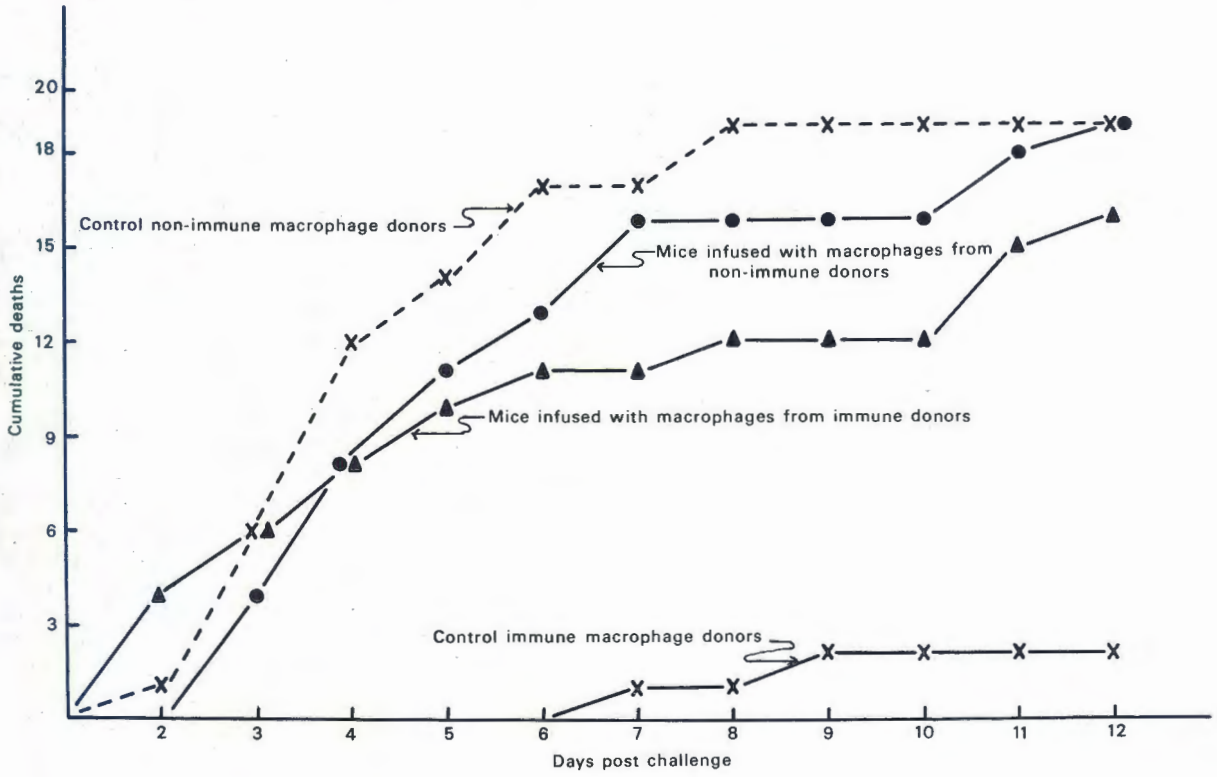

FIG. 3   Effect of passively transferred macrophages on resistance. Recipient mice were infused with macrophages from four donors.
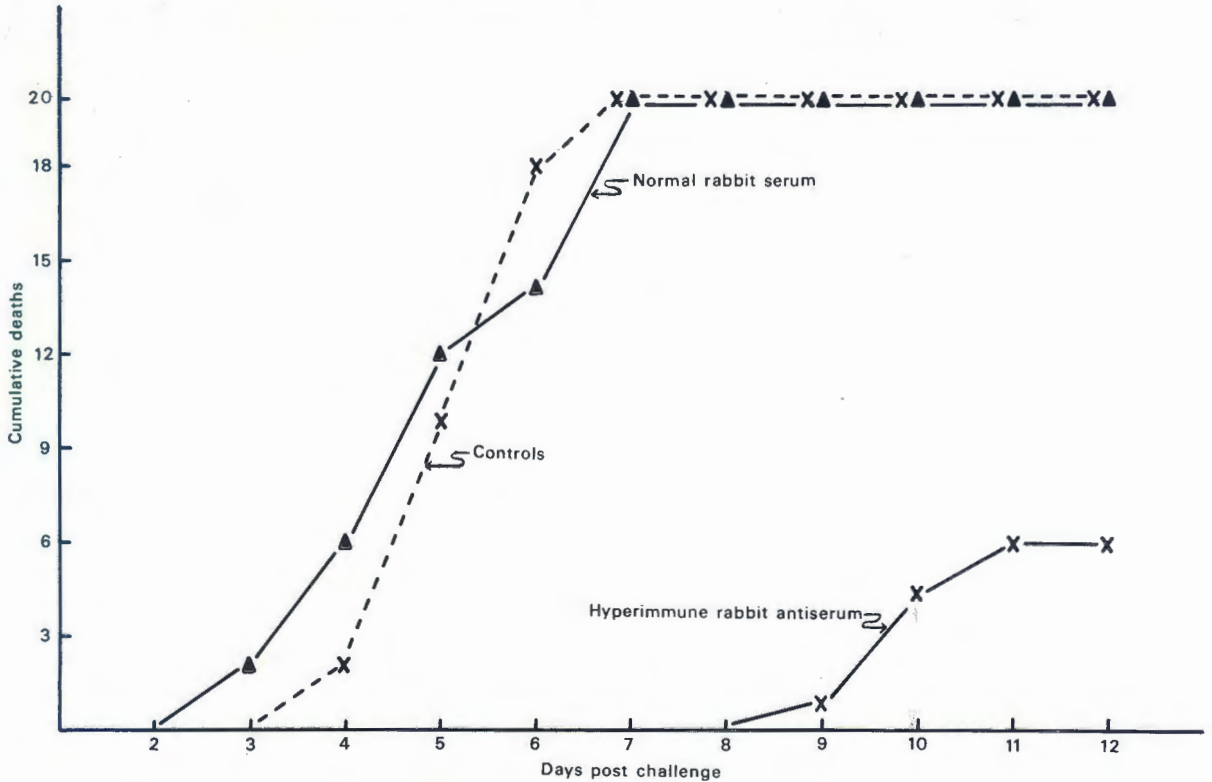

FIG. 4   Protective effect of hyperimmune whole cell rabbit antiserum against subacute infection. Mice received 0,2 ml serum intravenously and were challenged with 10^6 live bacteria intravenously.

actively immunized mice, the difference between the two groups is negligible and any advantageous effect that may be present is non-specific and can be accounted for by the large number of transferred cells.

On the other hand the intravenous administration of hyperimmune serum had a marked protective effect as shown in Fig. 4. Jolly (1965b) has shown that the pathogenesis of acute or subacute infections of *C.*
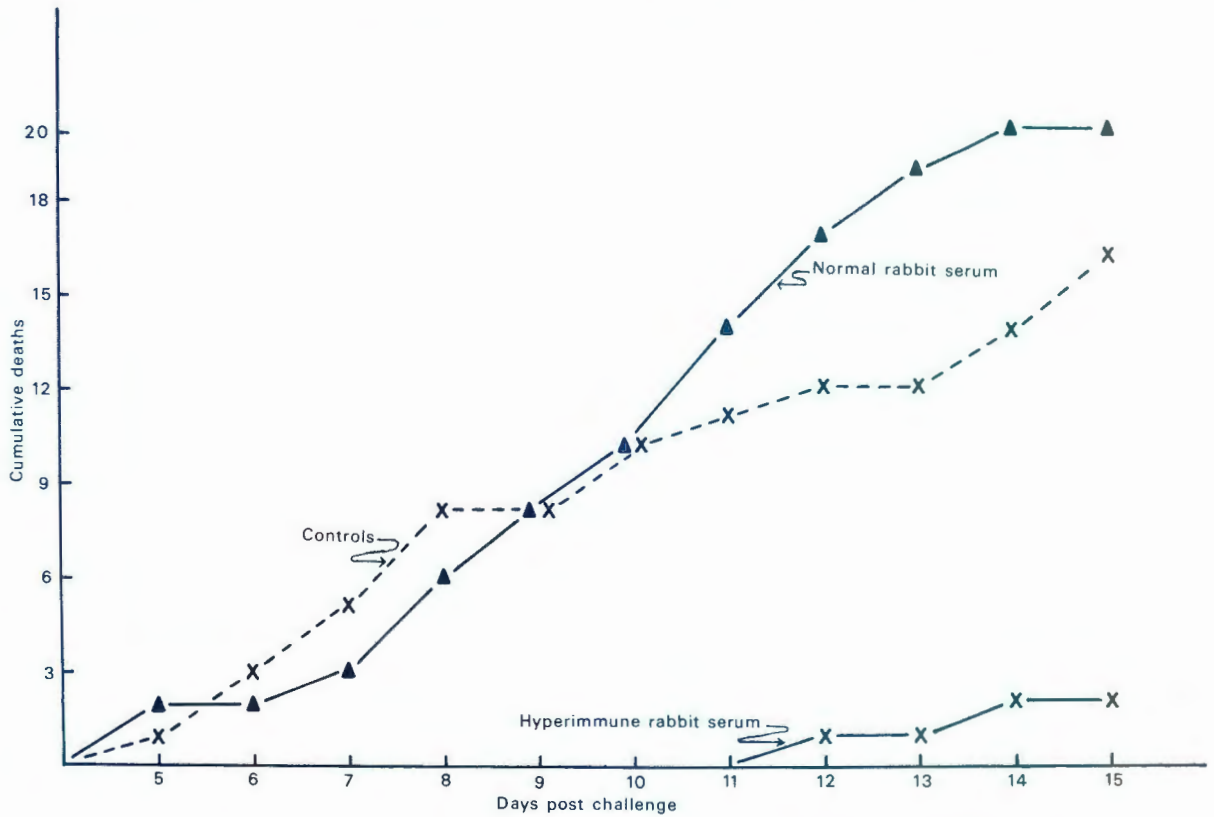

FIG. 5   Protective effect of hyperimmune whole cell rabbit antiserum against chronic infection. Mice received 0,2 ml serum intravenously and were challenged with 4×10^4 live bacteria intravenously.
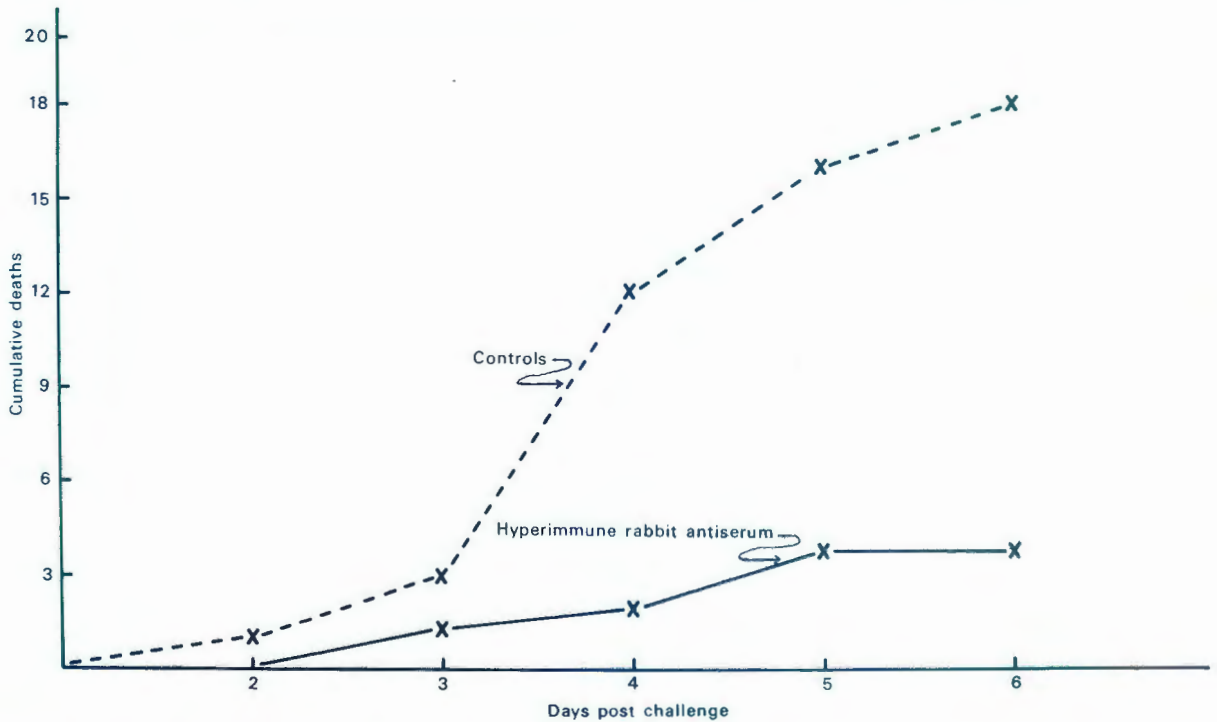

FIG. 6   Protective effect of hyperimmune whole cell rabbit antiserum against intraperitoneal infection. Mice received 0,2 ml serum intraperitoneally and were challenged with 2×10^6 live bacteria intraperitoneally.

pseudotuberculosis differs from that of chronic infections. Since a high challenge dose (10^6 bacteria) is considered as representative of an acute infection, the protective activity of antiserum was also determined for a lower challenge dose which is representative of a chronic infection. When passively immunized mice were challenged with 4×10^4 bacteria they also exhibited a marked degree of immunity (Fig. 5).

For further studies on the mechanism of immunity it was necessary to follow the bacterial population changes during the course of infection and this necessitated the intraperitoneal infection of mice. It would have been preferable to use doses in the range of 2×10^5 or 4×10^4 bacteria, which give rise to subacute or chronic infections when given intravenously, but these dosages via the intraperitoneal route would not consistently infect a satisfactory number of animals. Consequently higher doses had to be employed and 2×10^6 live bacteria were found to be the most satisfactory. As shown in Fig. 6, hyperimmune serum administered intraperitoneally protected against this challenge dose, though it would not protect against 10^7 bacteria.

One advantage of a challenge dose of 2×10^6 was that there was much less variation in the bacterial populations in the peritoneum of individual mice compared to the variations encountered in mice infected with 10^6, 2×10^5 or 4×10^4 bacteria.

Figure 7 shows the bacterial population changes in actively immunized mice following intraperitoneal challenge. There was virtually no difference between immunized and control groups during the first 6 hours after infection: the number of organisms decreased in both groups at approximately the same rate. Thereafter, however, the numbers continued to decline in the immunized group while they rapidly increased in the control group. Thus, although the initial rate of destruction of bacteria is not enhanced in immunized mice, the subsequent multiplication of bacteria is prevented in them.

Essentially the same pattern was found in mice which were passively protected with antiserum, but the difference between the two groups was not as marked. Both groups eliminated the bacteria equally well during the first 12 hours after infection, but secondary multiplication was inhibited in the immunized mice and not in the control group.
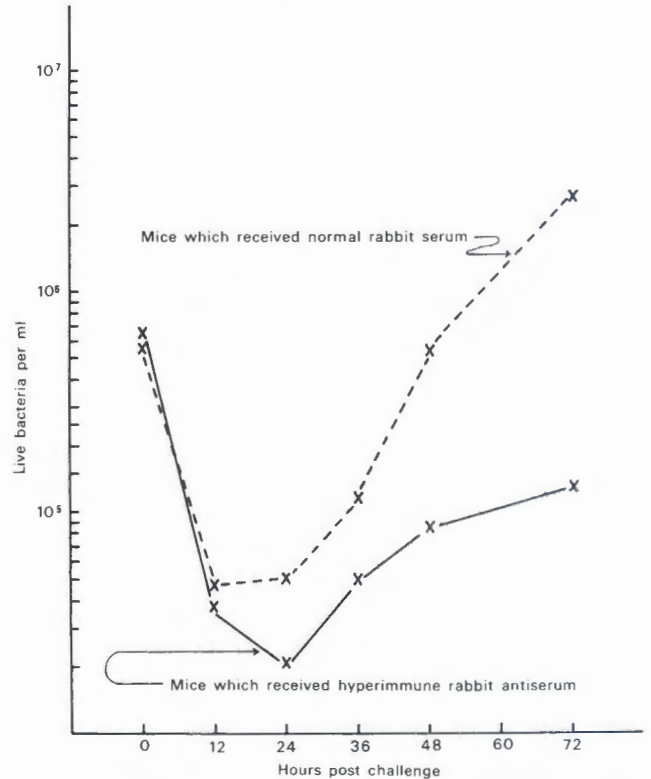
FIG. 8 Bacterial population changes in the peritoneum of mice which had received hyperimmune or normal rabbit serum. Mice were given 0,2 ml serum intraperitoneally and challenged with 2×10^6 live bacteria intraperitoneally.

In the following experiment the influence of the interval between administration of antiserum and challenge as well as preopsonization was studied.

Neither mice which were given antiserum 18 hours after challenge nor mice which were challenged with opsonized bacteria were protected. However, those which were given antiserum 18 hours before challenge or simultaneously with the challenge dose were protected.

These findings indicate that immunity was not due to opsonization, phagocytosis and immediate destruction of the infecting bacteria, but to inhibition or retardation of the secondary multiplication of the organisms in either actively or passively immunized animals.

One possible explanation is that death followed the liberation of exotoxin by the multiplying bacteria and that the protective effect of the antiserum was due to its antitoxic activity.

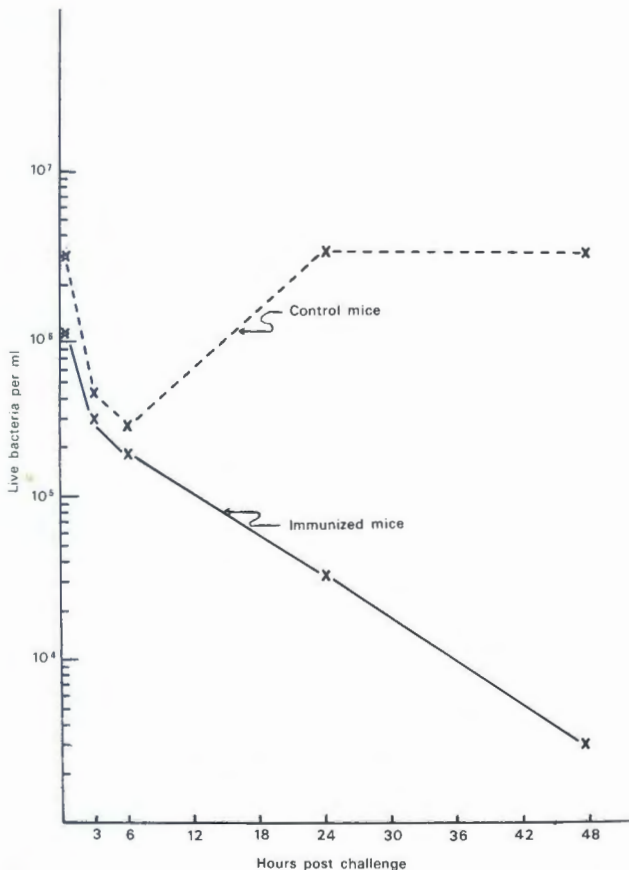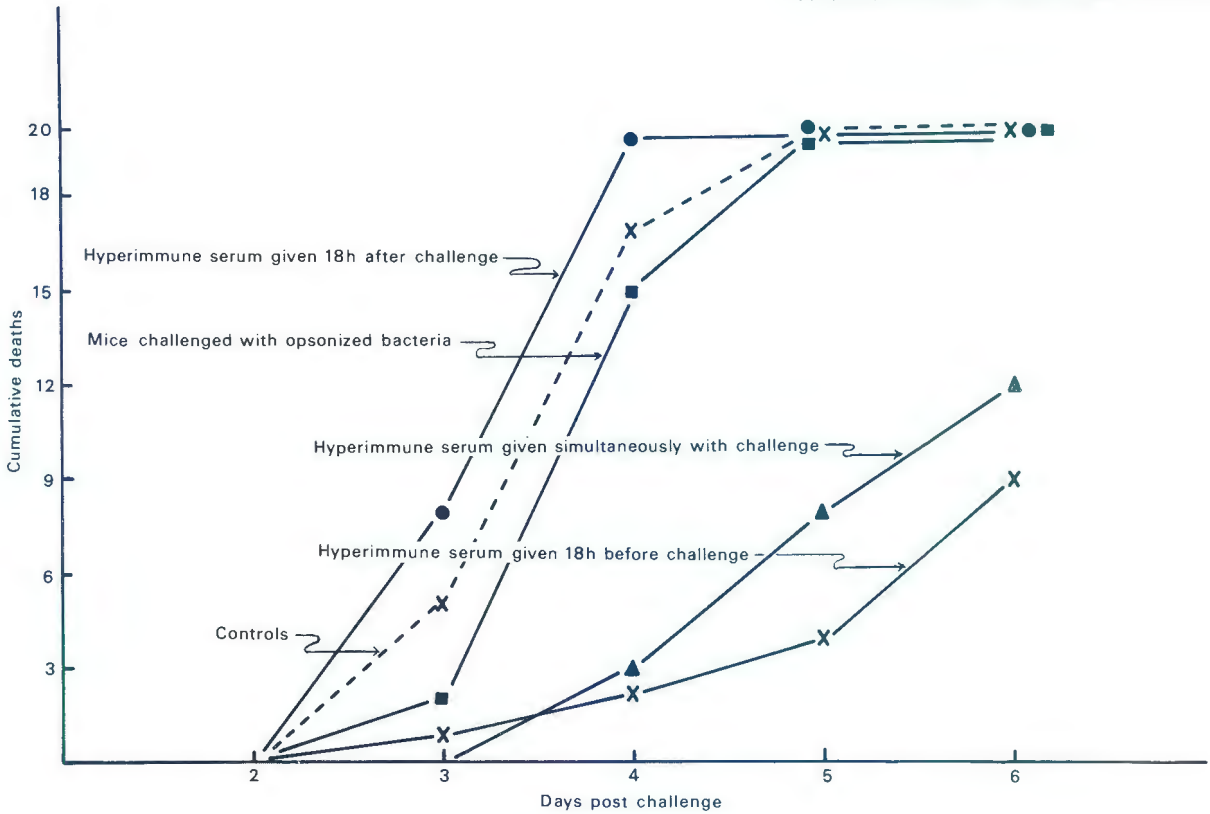In order to elucidate this theory, the protective activity of the HRA was compared with that of an

FIG. 7 Bacterial population changes in the peritoneum of actively immunized mice. Mice were immunized with two injections of aluminium phosphate vaccine (1,0 mg per dose) and challenged with 10^7 live bacteria intraperitoneally.

FIG. 9 Protective effect of hyperimmune antiserum given at different times relative to time of challenge. Mice received 0,2 ml serum intravenously and were challenged with 10^6 live bacteria intravenously.
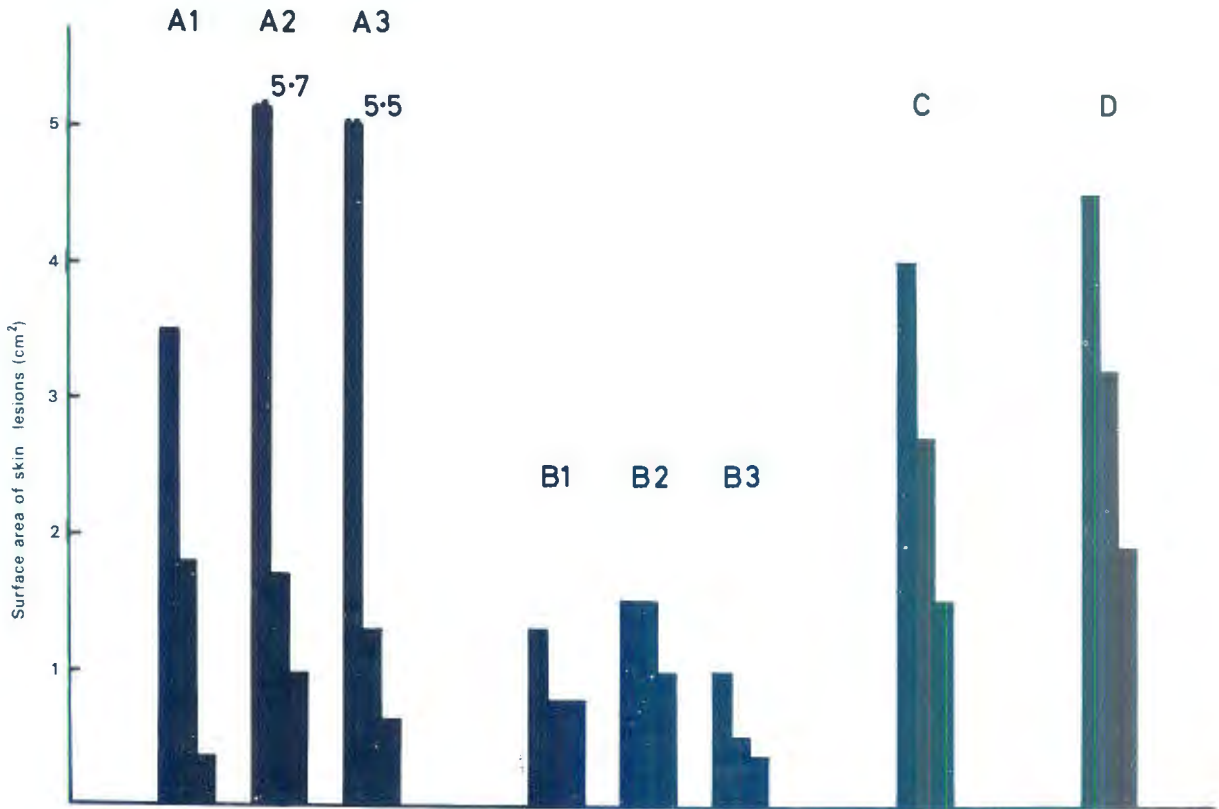

FIG. 10 Antitoxic activity of antisera
A 1, A 2 & A 3 = Hyperimmune whole cell antisera
B 1, B 2 & B 3 = Anti-exotoxin
C   = Normal rabbit serum
D   = Toxin control
Each bar represents the effect of the serum on undiluted toxin, $\frac{1}{5}$ and $\frac{1}{25}$ dilutions of toxin.
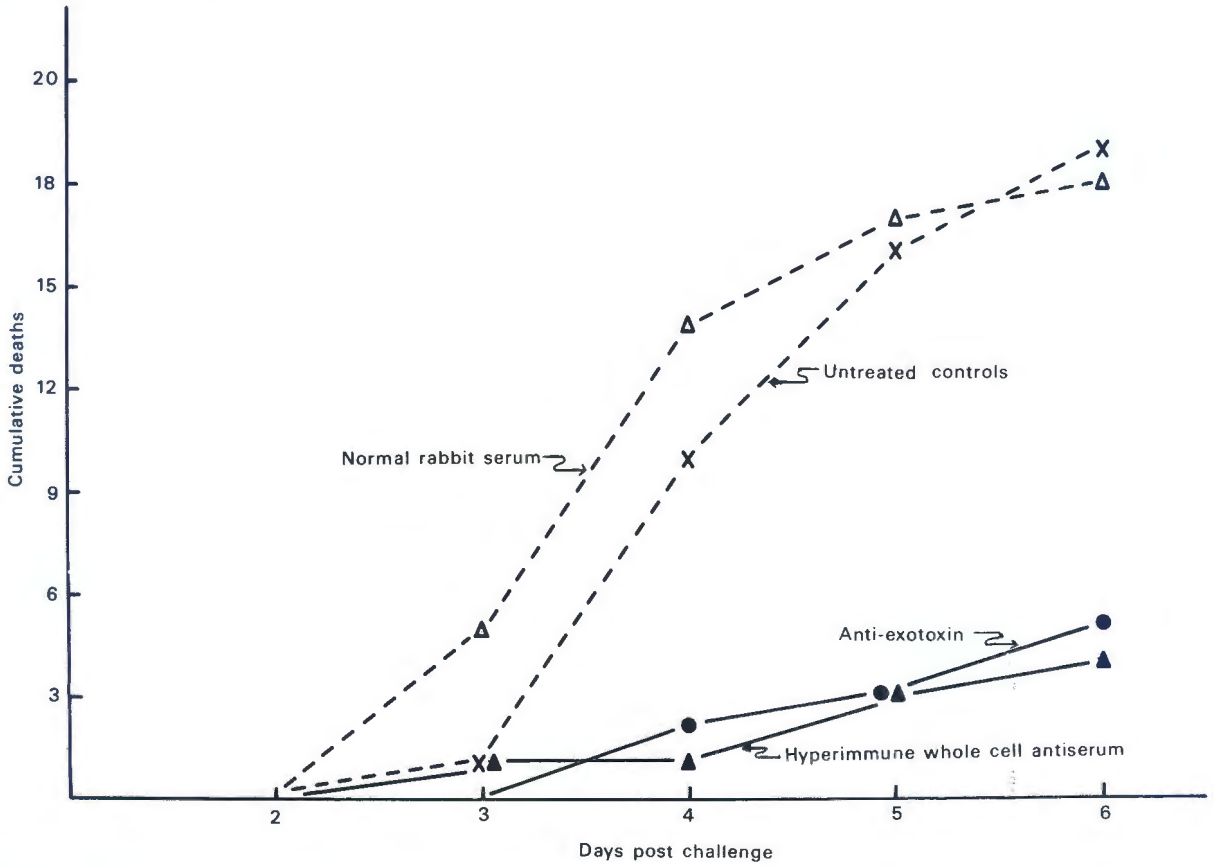
FIG. 11 Protective effect of hyperimmune whole cell antiserum and anti-exotoxin. Mice received 0,2 ml serum intravenously and were challenged with 10⁶ live bacteria intravenously.
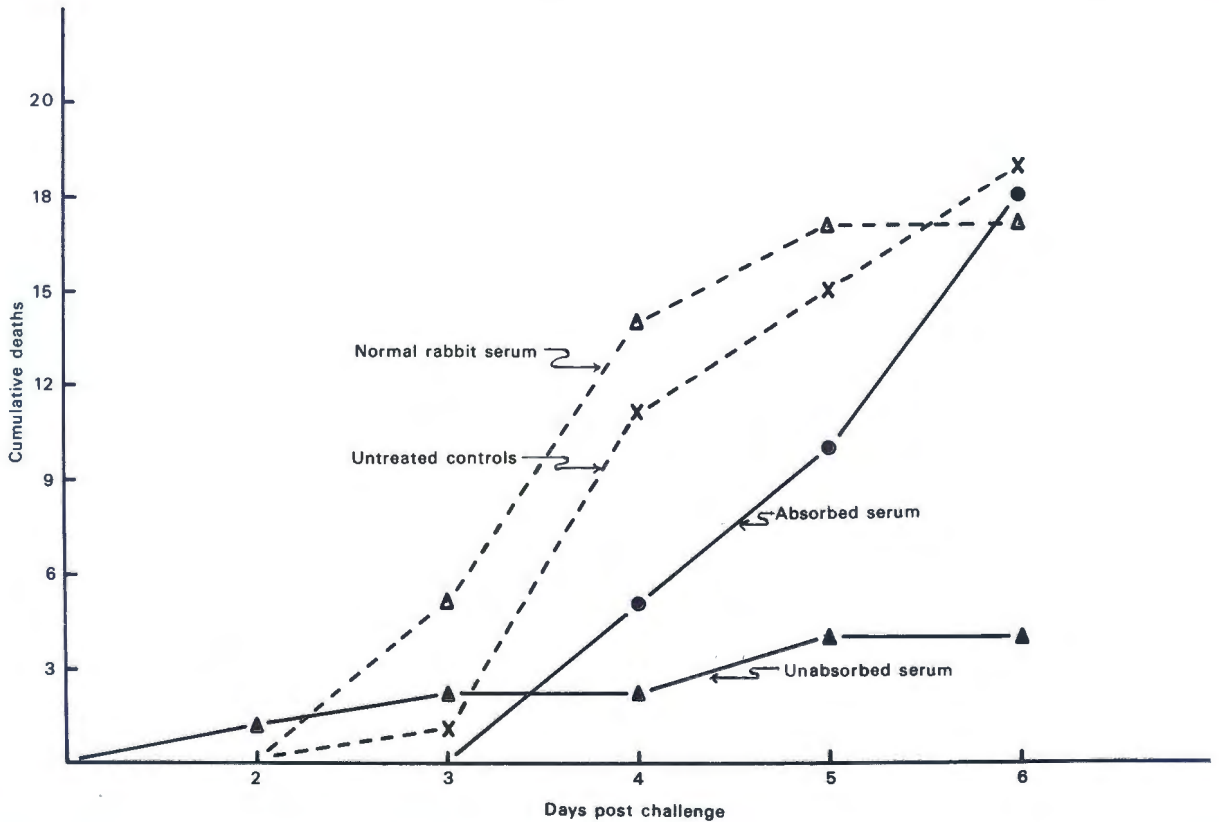

FIG. 12 Reduction of protective effect of hyperimmune whole cell antiserum after absorption with heated bacteria. Mice received 0,2 ml serum intravenously and were challenged with 10⁶ live bacteria intravenously.

antiserum prepared against exotoxin. Both the HRA and the antitoxin had agglutination titres of $\frac{1}{320}$, but there was a marked difference in their toxin neutralizing activity, as is clearly shown in Fig. 10.

The HRA which was devoid of antitoxic activity protected mice just as well as did the antitoxin (Fig. 11). The protective activity of the antitoxin can readily be accounted for by virtue of its antibacterial antibodies, as reflected by the agglutination titre.

Another possible explanation was that the hyperimmune serum contained an antibacterial factor which retards multiplication of the organisms but is not involved in opsonization and phagocytosis. This was proved by testing the protective effect of HRA absorbed with fresh bacteria; after absorption the agglutination titre was decreased from $\frac{1}{320}$ to $\frac{1}{40}$ and the protective activity of the serum was markedly reduced (Fig. 12).

DISCUSSION

The mechanisms of immunity to *C. pseudotuberculosis* have not been studied in great detail, primarily because of the complexity of the natural disease in sheep and the difficulty of reproducing the disease experimentally in this species.

At present the only alternative is to use mice as an experimental model, but Jolly (1965b) has shown that the pathogenesis, and probably also the mechanism of immunity, may differ depending on the intensity of infection.

According to Jolly (1965a), the exotoxin plays an important role in the pathogenesis of *C. pseudotuberculosis* and antitoxin may therefore be of some protective value, particularly in acute infections.

However, the natural disease is chronic in nature and it is doubtful if antitoxin is of much use in such cases. Furthermore, Cameron & Minnaar (1969) and Cameron *et al.* (1969) have shown that mice can be immunized with intact dead bacteria as well as with cell walls. Considerable circumstantial evidence has been provided by Cameron & Smit (1970) that anti-exotoxin is probably of little consequence in immunity.

Data are also presented in this paper showing that antibacterial antiserum which is devoid of antitoxic activity is quite capable of passively protecting mice.

Initially it was thought that, despite previous findings, a solid immunity could not be obtained unless a live vaccine is used. Such a vaccine would possibly induce a cellular as well as a humoral immunity and cumulatively these would afford a more substantial resistance to infection. However, it was found that any lack of immunity can be overcome by administering an adequate amount of antigen. The use of a live vaccine is therefore not essential in order to obtain a good immunity.

Comparison of the passive immunizing properties of HRA and macrophages from immune mice showed that at best the latter had only slight activity, while the serum conferred a marked degree of immunity on the recipient mice. Jolly (1965c) and Hard (1969) have presented evidence to the contrary but both these authors used live organisms to immunize their mice and, as pointed out by Hard (1969), the activity of the macrophages is primarily of a non-specific nature.

A study of the population dynamics of bacteria in the peritoneal cavity of actively and passively immunized mice revealed certain interesting features. The outstanding observation was that initially the decrease in the total number of bacteria was the same in immune and in normal animals. This finding indicated that resistance to infection is not due to enhanced phagocytosis and destruction of bacteria. This deduction was substantiated by further experiments in which it was shown that pre-opsonization of bacteria with HRA did not reduce their invasive and infective properties.

The resistance of immunized mice was due to their ability to retard and restrict the secondary multiplication of bacteria. This property of HRA is not due to antitoxic activity and can be abolished by absorption with whole bacteria.

It is thus apparent that immunity to *C. pseudotuberculosis* is primarily of a humoral nature and that when an inactivated vaccine is used cellular immunity is negligible. However, this does not necessarily exclude the possible role of cellular immunity if a live vaccine is employed, or its potential role in other species such as the sheep.

SUMMARY

Experimental evidence is presented to show that immunity to *C. pseudotuberculosis* in mice is of a humoral nature when an inactivated vaccine is employed.

Macrophages from immune mice have no protective properties, but mice can be immunized passively with hyperimmune rabbit antiserum.

The protective effect of the serum is not due to antitoxic activity or its ability to promote phagocytosis. Immunity is due to retarded secondary multiplication of bacteria, a phenomenon which was observed in both actively and passively immunized mice. This property can be removed from hyperimmune serum by absorption with whole bacteria.

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