

IMMUNIZATION OF MICE AGAINST *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* INFECTION

C. M. CAMERON and J. L. MINNAAR, Veterinary Research Institute, Onderstepoort

ABSTRACT

CAMERON, C. M. & MINNAAR, J. L., 1969. Immunization of mice against *Corynebacterium pseudotuberculosis* (Buchanan, 1911) infection in mice. *Onderstepoort J. vet. Res.*, 36 (2), 207-210 (1969).

Mice were immunized with vaccines containing whole dry bacteria and adjuvant. They were challenged by the intravenous injection of living bacteria. The degree of immunity obtained was best expressed in terms of the difference in the Tangens values of the cumulative death rates between the immunized and control animals.

INTRODUCTION

Literature on the development of immunity to *Corynebacterium pseudotuberculosis* (Buchanan, 1911) infections is extremely sparse. In 1934 Petrie & McClean reported that they were able to immunize horses and guinea pigs by administering repeated large doses of toxoid and formalin killed bacteria. Cameron (1964) & Cameron & Buchan (1966) had some success in guinea pigs when using formalized or phenolized cells. Carne, Wickham & Kater (1956) studied the immunizing properties of the exotoxin and found that although it stimulates the production of antibodies which would protect experimental animals against lethal doses of exotoxin, antitoxic immunity would not protect against infection with live organisms.

The only other recent reports on immunity to *C. pseudotuberculosis* are those by Jolly (1965a; 1965c). He demonstrated that the lymph nodes of sheep which had received repeated doses of toxoid and exotoxin were resistant to infection. In a series of experiments he also demonstrated that convalescent mice developed a resistance to reinfection.

In our studies on immunity to *C. pseudotuberculosis* one of the major problems has been the lack of a suitable test system. The use of guinea pigs gave fair results but a quantitative evaluation of the infection in immunized and control groups was difficult. A more accurate assay method is essential, particularly because the degree of immunity thus far obtained has been of a low order.

An attempt was, therefore, made to determine whether the mouse system used by Jolly (1965b) could be adapted as a standard method for the evaluation of vaccines and other immunological experiments.

In the experiments described here mice were immunized with formalized corynebacterial suspensions to which various adjuvants had been added. They were subsequently challenged by the intravenous administration of pathogenic bacteria. They were destroyed at various intervals and the number of bacteria contained in their spleens was determined.

MATERIALS AND METHODS

Infection of experimental mice

The strain used in all the experiments was No. 137B which was originally obtained from Prof. Carne of the University of Sydney. The bacteria were grown in Roux flasks, which were continuously shaken, in

a medium described by Cameron & Swart (1965). The cultures were allowed to stand for 30 minutes to permit sedimentation of all coarse particles. The homogenous upper portion of the culture was decanted and centrifuged. After discarding the medium, the concentrated cells were resuspended in half the original volume of medium in a buffer solution consisting of 5 per cent lactose and 1 per cent Difco peptone in 0.02M phosphate buffer pH 7.2 to 7.4. After two to three days storage at -20°C , counts to determine the viability of the bacteria were done by plating on blood tryptose agar (Difco).

For pathogenicity determinations and challenge of immunized animals, the frozen cultures were diluted in saline to give the required number of live organisms per ml. All challenge injections were done intravenously in the tail vein and a volume of 0.2 ml was used throughout.

Preparation of vaccines

C. pseudotuberculosis strain 137B was also used for preparation of vaccine. Large volumes of cells were produced in liquid medium in static Roux flasks by the method described previously (Cameron & Swart, 1965). In order to obtain good pellicle development and the maximal yield, it was found important not to subject the medium to excessive sterilization.

The bacterial cells were harvested by centrifugation, washed twice in distilled water and lyophilized.

Vaccines were prepared by suspending the required dry weight of bacteria in 0.85 per cent saline containing 0.5 per cent formalin. The suspension was allowed to stand at room temperature overnight and the respective adjuvants were then added.

Vaccines for use in sheep were similarly prepared except that the cells were not harvested and dried but 0.5 per cent formalin added directly to the culture. The packed cell volume was determined and the density adjusted with 0.85 per cent saline according to the requirements of the particular experiment.

Adjuvants

Claassen's oil adjuvants was prepared from a light mineral oil and appropriate emulsifiers (N. Claassen, Veterinary Research Institute, Onderstepoort, personal communication, 1969).

Aluminium phosphate gel was prepared as described by Sterne & Wentzel (1950).

IMMUNIZATION OF MICE AGAINST *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* INFECTION

Freund's complete and incomplete adjuvant* and Alhydrogel** were used as supplied by the manufacturers.

Immunization and challenge of mice

Four weeks old female Swiss Webster albino mice weighing between 17 and 22 g were used in all the experiments. Each animal received two injections of 0.2 ml vaccine subcutaneously at an interval of three weeks.

Three groups of mice were used for each dose of every vaccine to be tested. The three groups were challenged 10 days after the second injection with 10^6 , 2×10^5 and 4×10^4 bacteria respectively.

In initial experiments the total number of bacteria in the individual spleens of the surviving mice was counted seven days after challenge. The mice were killed with chloroform and their spleens homogenized in 0.9 ml sterile saline (1:10 w/v dilution). Tenfold dilutions were prepared and counts of live bacteria were made by spreading 0.1 ml of each dilution on blood tryptose agar plates. Colonies were counted after incubation at 37°C for 48 hours.

RESULTS

Enumeration of live bacteria

Initially the medium used for vaccine production, to which 1.5 per cent agar had been added, was used for plate counts. It was, however, found that although good growth was obtained at low dilutions, it did not consistently support the development of single colonies at the higher dilutions. Consequently, blood tryptose agar plates (Difco) were used for this purpose.

A further complicating factor was the difficulty encountered in interpreting the results due to a considerable variation in the number of bacteria in the spleens of individual mice. It was, however, found that if a subacute infection could be established with death occurring after 4 to 14 days, interpretation of the results was facilitated by recording the cumulative death rates.

A dose of 10^6 live bacteria per mouse usually gave rise to an infection rate that was suitable for spleen count assays, while a dose of 2×10^5 or 4×10^4 bacteria per mouse gave more satisfactory results when it was desired to record the cumulative deaths.

Variations in virulence

During the course of this study it was observed that a given number of live bacteria did not consistently produce the same degree of infection in the mice. This was initially thought to be due to a difference in pathogenicity between organisms derived from either large or small colonies but pathogenicity tests in mice showed that subcultures from large and small colonies were equally virulent.

An experiment was therefore conducted to determine the effect of storage at -20°C on the virulence of the organisms. The counts of live bacteria remained constant for two months but the results in Table 1 clearly show that there was a marked drop in the virulence of the organisms during this period.

To obviate this problem to some extent, bacteria were never stored in the frozen state for longer than four weeks.

TABLE 1.—Virulence of *C. pseudotuberculosis* stored at -20°C.

Dose live bacteria per mouse	Cumulative deaths/10 mice								
	10^6			2×10^5			4×10^4		
Storage time days	2	30	60	2	30	60	2	30	60
Day 1	0	0	0	0	0	0	0	0	0
Day 2	1	3	0	0	0	0	0	0	0
Day 3	8	6	1	1	0	0	0	0	0
Day 4	10	9	3	5	1	0	0	0	0
Day 5		10	5	6	3	0	2	0	0
Day 6			5	9	7	1	5	1	0
Day 7			5	10	9	4	7	2	0
Day 8			5		10	4	10	7	0
Day 9			5			4		9	1
Day 10			6			5		10	2

Estimation of minimum immunizing dose

Vaccine was prepared as described above, emulsified with Freund's complete adjuvant and groups of mice immunized. In this experiment the results recorded in Fig. 1 are of the mice which were challenged with 4×10^4 recently frozen bacteria. Two doses of 1.0 mg conferred a solid immunity and resistance could even be demonstrated with as little as 0.04 mg antigen per dose. Freund's complete adjuvant alone had virtually no effect on susceptibility.

Comparison of adjuvants

Groups of 24 mice were immunized with vaccines containing Freund's complete adjuvant, Freund's incomplete adjuvant and Alhydrogel. The vaccines were prepared by mixing equal volumes of adjuvant and bacterial suspension. Each 0.2 ml dose contained 1.0 mg dry bacteria.

The cumulative deaths of mice which were challenged with 2×10^5 bacteria are shown in Fig. 2.

The mice in the immunized groups were much more resistant to challenge than the control group. There was, however, no significant difference between the three adjuvants. This was also reflected in the Tangens values of the respective graphs. The values obtained were 0.8 for the control group, 0.25 for the complete and incomplete Freund groups and 0.2 for the Alhydrogel group.

Mice were also immunized with vaccines which contained no adjuvants but inconsistent results were obtained.

Potency test of vaccines suitable for use in sheep

Previous experiments had indicated that 5 ml of a vaccine containing 0.5 per cent packed cells will give an optimal agglutinin response in sheep. Three vaccines were therefore prepared as described above

(*) Difco Laboratories, Detroit, Michigan, U.S.A.

(**) Dansk, Svovlsyre, Denmark

and the density adjusted to give the desired concentration per sheep dose. The composition of the three adjuvant vaccines is give in Table 2.

TABLE 2.—Composition of sheep vaccines

Adjuvant	Pack- ed cell vol- ume	Ratio Ad- juvant: Cell sus- pension	Propo- sed sheep dose	Dose per mouse	Dry weight of bac- teria per mouse dose
	%		ml	ml	mg
Claassen oil adjuvant	1.0	1:1	5	0.2	0.1
Aluminium phosphate gel	1.0	1:1	5	0.2	0.1
8% Potassium alum solution	1.7	1:3	2	0.1	0.12

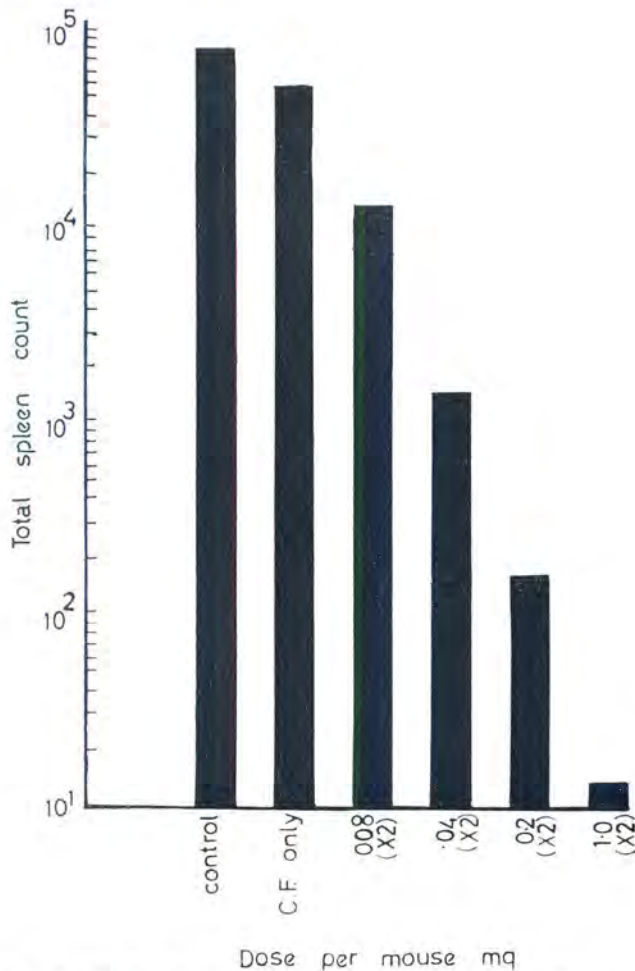


FIG. 1.—Mean bacteria per spleen of mice given different doses of antigen with Freund's complete adjuvant and challenged with 4×10^4 live bacteria per mouse

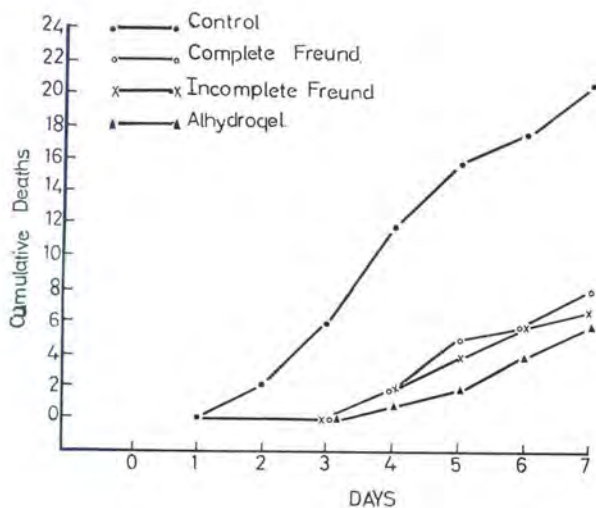


FIG. 2.—Cumulative deaths of mice immunized with vaccines containing different adjuvants and challenged with 2×10^5 live bacteria per mouse

Two injections of vaccine were administered to mice according to the standard schedule. The cumulative deaths after challenge are shown in Fig. 3.

The good protection is also evident when the Tangens values of the graphs are compared. The values obtained were 0.66, 0.16, 0.16 and 0.05 for the control, alum, aluminium phosphate and oil adjuvant groups respectively. This gives a difference of at least 0.5 between the control and all immunized groups.

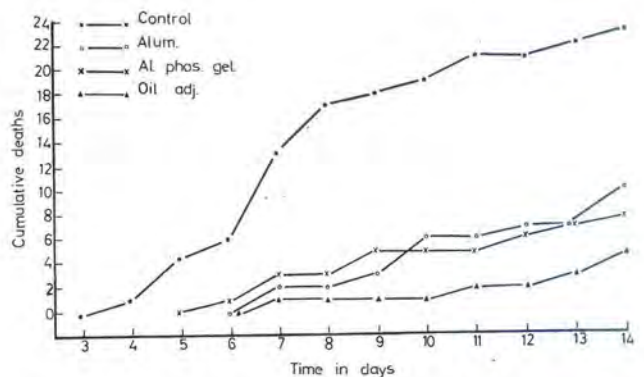


FIG. 3.—Cumulative deaths of mice which had received three different sheep vaccines and which were challenged with 4×10^4 live bacteria per mouse

DISCUSSION

The data presented in this paper show that it is possible to immunize mice with formalized bacteria and that the increase in resistance can be clearly demonstrated by recording the cumulative death rates. It is, however, difficult to consistently reproduce a precise death rate in the control groups in every experiment. Consequently, the slope of the graph varies from one experiment to another and makes comparison difficult. This problem can be overcome by calculating the Tangens value of the slopes and expressing the degree of resistance as the difference in the Tangens values between the immunized and control groups.

C. pseudotuberculosis produces a lethal exotoxin (Carne, 1940; Lovell & Zaki, 1966) which contributes considerably towards the pathogenesis of the infection (Jolly, 1965a). Our results indicate that because a good immunity could be obtained with washed whole cells alone, antitoxin probably plays a very minor role in conferring immunity to infection with living bacteria.

It has nevertheless been shown by Jolly (1965b) that there is a difference in the pathogenesis in acute and subacute infections in mice. In acute cases antitoxin may be beneficial, but because the disease in sheep is generally very chronic this facet of immunity is probably of little consequence in practice.

The mechanism of immunity is not yet clearly defined. Jolly (1965c) is of opinion that immunity results from an increase in the antibacterial activity of macrophages. We have, however, been able to transfer passive immunity by means of serum alone which suggests that immunity is mediated primarily through serum antibodies and a process of enhanced opsonization.

SUMMARY

Mice were successfully immunized against subacute infections with *C. pseudotuberculosis*, using vaccines containing whole bacteria and adjuvant. Adjuvant vaccines suitable for administration to sheep were also shown to produce a satisfactory immunity in mice. Immunization with two doses of either 0.1, 0.2 or 1.0 mg of dry bacteria per mouse gave equally satisfactory results. Calculation of the Tangens values of the cumulative death rates, gave a difference of at least 0.5 between the immunized and control groups in all instances.

ACKNOWLEDGEMENTS

We wish to thank Dr. R. C. Tustin and Dr. R. D. Bigalke for their assistance in compiling this paper.

Thanks are due to Miss M. Smit and the section of Photography for drawing and reproducing the graphs, and Mr. N. Claassen for composing the oil adjuvant.

REFERENCES

- CAMERON, C. M., 1964. The significance of the endotoxin and pyogenic factor of *Corynebacterium pseudotuberculosis* in immunity. *Onderstepoort J. vet. Res.*, 31, 119-132.
- CAMERON, C. M. & BUCHAN, L., 1966. Identification of the protective and toxic antigens of *Corynebacterium pseudotuberculosis*. *Onderstepoort J. vet. Res.*, 33, 39-48.
- CAMERON, C. M. & SWART, C. F., 1965. A new liquid medium for the cultivation of *Corynebacterium pseudotuberculosis*. *Jl S. Afr. vet. med. Ass.*, 36, 185-188.
- CARNE, H. R., 1940. The toxin of *Corynebacterium ovis*. *J. Path. Bact.*, 51, 199-212.
- CARNE, H. R., WICKHAM, N. & KATER, J. C., 1956. A toxic lipid from the surface of *Corynebacterium ovis*. *Nature, Lond.*, 178, 701-702.
- JOLLY, R. D., 1965a. The pathogenic action of the exotoxin of *Corynebacterium ovis*. *J. comp. Path.*, 75, 417-431.
- JOLLY, R. D., 1965b. The pathogenesis of experimental *Corynebacterium ovis* infection in mice. *N. Z. vet. J.*, 13, 141-147.
- JOLLY, R. D., 1965c. Experimental infection of convalescent mice with *Corynebacterium ovis*. *N.Z. vet. J.*, 13, 148-153.
- LOVELL, R. & ZAKI, M. M., 1966. Studies on the growth products of *Corynebacterium ovis*. I. The exotoxin and its lethal action on white mice. *Res. vet. Sci.*, 7, 302-306.
- PETRIE, G. F. & McLEAN, D., 1934. The interrelationship of *C. ovis*, *C. diphtheriae*, and certain diphtheroid strains from the human nasopharynx. *J. Path. Bact.*, 39, 635-663.
- STERNE, M. & WENTZEL, L. M., 1950. A new method for the large-scale production of high-titre botulinum formal-toxoid types C and D. *J. Immun.*, 65, 175-183.