Onderstepoort J. vet. Res. (1969), 36 (2), 211–216
Printed in the Republic of South Africa by T. W. Hayne Ltd. (H. & G.), Johannesburg, for the Government Printer, Pretoria

IMMUNIZING PROPERTIES OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* CELL WALLS

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

ABSTRACT

CAMERON, C. M., MINNAAR, J. L. & PURDOM, MARY R., 1969. Immunizing properties of *Corynebacterium pseudotuberculosis* (Buchanan, 1911) cell walls. *Onderstepoort J. vet. Res.*, 36 (2), 211—116.

Mice were successfully immunized with purified cell walls. The immunizing properties were not affected by extraction with ether: ethanol or trichloracetic acid but were destroyed after treatment with formamide.

INTRODUCTION

The absence of a serological test which could be used as a measure for protective immunity, is a major obstacle in the development and evaluation of an effective vaccine for immunization of sheep against infection with *Corynebacterium pseudotuberculosis* (Buchanan, 1911). In order to overcome this problem a study was undertaken to determine which antigen is responsible for inducing a protective immunity.

In a recent paper Cameron & Minnaar (1969) showed that mice could be satisfactorily immunized with formalized whole bacteria injected together with an adjuvant. It now became necessary to find which portion of the organism was concerned in this process. The immunizing properties of the cell walls and protoplasm were first compared and, when it became apparent that the cell walls were more potent, they were examined in greater detail.

Whole bacteria were mechanically ruptured and the cell walls subjected to standard purification procedures. In order to gain further information on the nature of the antigen, enzymatically purified cell walls were subjected to extraction procedures which are commonly used in the study of cell wall antigens of Gram positive bacteria. Ether: ethanol is known to remove the loosely bound surface lipid of *C. pseudotuberculosis* (Bull & Dickinson, 1935; Jolly, 1966) and extraction with trichloracetic acid (TCA) is a recognised procedure for removing teichoic acids (Amstrong, Baddily & Buchanan, 1960; Morse, 1962). Formamide has been used to prepare polysaccharide antigens from streptococci (Krause & McCarth, 1961; Heyman, Manniello & Barkulis, 1963) and *Propionibacterium* spp. (Allsop & Work, 1963). These procedures were applied to *C. pseudotuberculosis* cell walls.

The ability of the cell walls to induce protection in mice as well as their chemical composition was determined after each step in the purification and extraction process.

MATERIALS AND METHODS

Production of bacterial cells

C. pseudotuberculosis strain 137B was used in all the experiments. Masses of cells were produced in static cultures as previously described (Cameron & Swart, 1965). The cells were harvested by centrifugation, washed twice with distilled water and stored at −20°C.

Preparation and purification of cell walls

The method used for breaking the cells was based on the procedure described by Bleiweis, Karakawa & Krause (1964). Twenty grams of packed cells were suspended in 80 ml 0.05 M phosphate buffer (pH 8.0). The suspension was divided into three portions and each portion mixed with 30 ml glass beads (0.17 mm diam.). The portions were shaken in a Braun homogenizer model MSK* at 4,000 rpm for 5 min taking care to keep the chamber cool.

After disruption the portions were pooled, diluted with 90 ml cold distilled water and the beads allowed to settle out. The cell walls and unbroken cells were separated from the protoplasm by centrifugation at 40,000 *g* for 30 min at 4°C.

The sediment was resuspended in 180 ml buffer and the unbroken cells and cell walls were separated by a modification of a technique described by Ribi & Hoyer (1960). Linear sucrose gradients (200 ml, 0 to 40 per cent) were prepared in 250 ml centrifuge bottles. Thirty millilitres of the suspension was layered on each bottle and centrifuged at 600 *g* for 1 hour. The cell wall layer was drawn off, one third volume of distilled water added and centrifuged at 20,000 *g* for 30 min at 4°C. The cell walls were washed twice with cold distilled water, suspended in distilled water and examined for purity by electron microscopy.

The cell walls were freed of residual protoplasm and ribonucleic acid by treatment with ribonuclease, trypsin and pepsin as described by Kwapinsky (1965).

Chemical treatment of cell walls

Enzymatically purified cell walls were successively extracted with ether: ethanol, TCA and formamide. Aliquots were collected after each stage, washed twice in distilled water, lyophilized, and used for chemical analysis and immunity experiments.

Ether: ethanol extraction was carried out by suspending the cell walls obtained from 20 g of wet cells in a mixture of 50 ml ether and 30 ml absolute ethanol and stirring for 18 hours at 4°C (Kwapinsky, 1965). Hereafter the cell walls were washed once in distilled water and extracted with 80 ml 10 per cent TCA for five days at 4°C. Extraction with formamide was done as described by Krause (1967).

Chemical analysis of cell walls

Protein was determined by Lowry's modification of the Folin-Ciocalteu method (cited by Kabat & Mayer, 1964). Five milligram samples were weighed out and

Received for publication on 21 August 1969. — Editor

(*) Bronwill Scientific, Rochester, N.Y., U.S.A.

10 ml of 1N NaOH added to each sample. The tubes were heated in boiling water for 10 minutes. The cell wall material did not go into suspension completely. One millilitre quantities of the boiled suspensions were used for testing.

For carbohydrate analysis it was found necessary to hydrolyze the cell wall material before testing. Samples were suspended in 0.1N H_2SO_4 to give 0.5 mg per ml, and heated at 60 °C for one hour. The cell wall material did not dissolve, but the particles became smaller, and a more representative sample could be obtained.

Hexoses were estimated by the anthrone reaction of Scott & Melvin (cited by Kabat & Mayer, 1964). Five millilitre volumes of the hydrolyzed suspensions were used. The material dissolved completely in the concentrated sulphuric acid of the anthrone reagent.

The Bial reaction (cited by Kabat & Mayer, 1964) was used to determine pentoses. One millilitre of the hydrolyzed suspension was diluted with 1.0 ml distilled water. Ferric chloride reagent (2 ml) was then added, followed by 0.2 ml of 10 per cent alcoholic solution of orcinol. The mixtures were heated in boiling water for 30 min in order to obtain full colour development. The cysteine HCl-sulphuric acid method of Dische (cited by Kabat & Mayer, 1964) was also employed to determine pentoses but was found to give variable results.

Phosphorus was determined by the ascorbic acid method of Chen, Toribara & Warner (1956). The wet ashing method did not give reliable results, so dry ashing was used. Ten milligrams samples were weighed out into crucibles, and heated in a furnace at approximately 500 °C for about an hour. Two millilitres of distilled water, and 1 ml of 1N HCl was added to the contents of each crucible. The solutions were gently evaporated to dryness, taking care to avoid spitting. The solids were then redissolved in distilled water, and the volumes made up to 25 ml, including 4 ml of test reagent, in volumetric flasks. The normal volume used in the test is 100 ml, but as only minute amounts of phosphorus were present, 25 ml was found to give better colour production.

N-acetyl hexosamine was estimated by the method of Reissig, Strominger & Leloir (1955). It was found necessary to pretreat the cell wall material to liberate the N-acetyl hexosamine. The required quantities of cell wall material (2.5 mg) were weighed out, and suspended in 3.5 ml of 0.01N HCl. The test tubes were plugged with cotton wool and placed in an oven at 100 °C for 10 min. The contents of the tubes were then evaporated to dryness in boiling water to remove the hydrochloric acid. The solids were resuspended in 2.5 ml distilled water and 0.5 ml aliquots were removed for testing as required. Each analysis on a particular sample was repeated three or four times in order to control the technique and obtain a reliable result.

PLATE 1.—Electronmicrograph of *C. pseudotuberculosis* cell walls

Immunity experiments

Each antigen was tested for immunizing activity by administering two injections of 1.0 mg, 0.2 mg and 0.04 mg respectively to three groups of ten mice. All the antigens were emulsified with an equal volume of Freund's complete adjuvant*. Control mice received Freund's complete adjuvant emulsified with saline. Later experiments were done in triplicate and each set of 30 mice was challenged with 10^6, 2×10^5 or 4×10^4 live bacteria. Immunity was assayed either by counting the total number of live bacteria per spleen 6 to 8 days after infection or by recording the cumulative deaths.

Details of the animals used, immunization schedules, preparation of challenge material, the technique used for counting the total number of bacteria per spleen and expression of the results in terms of the Tangens values of the cumulative death rates have been outlined in a previous paper (Cameron & Minnaar, 1969).

Results

Preparation of cell walls

Despite repeated efforts using a variety of buffers and procedures, whole cells and cell walls could not be satisfactorily separated by differential centrifugation. Intact cell walls invariably sedimented with the unbroken cells and showed a marked tendency to aggregate. On the other hand some of the unbroken cells became imbibed and floated to the surface. These problems could only be overcome by using a sucrose gradient. The whole cells sedimented to the bottom of the centrifuge bottles and the cell walls and cell wall fragments formed a well-defined band about one third down the gradient. As shown in Plate 1, the material obtained by this procedure was completely free from unbroken cells.

Immunizing properties of cell walls

The results of an experiment designed to determine which portion of the cell is responsible for inducing a protective immunity, are shown in Fig. 1. Whole cells gave the best results but were only slightly superior to untreated cell walls. It is doubtful whether this difference is of any significance. The degree of increased resistance produced by protoplasm can probably be accounted for by the presence of cell wall antigen which was liberated during the process of disruption.

Effect of chemical extraction procedures on the immunizing properties of cell walls

Enzymatically purified cell walls were successively extracted with ether: ethanol, TCA and formamide and the ability of the cell walls to induce protection was assayed in two experiments.

In the first experiment, the experimental mice were challenged with 2×10^5 live bacteria. The results of the total spleen counts of bacteria are shown in Table 1. Numerous mice died before the spleen counts were made. If these mice had survived, they would probably have had the highest number of bacteria per spleen. This would have influenced the final result considerably and given a much higher average count, particularly in the control and formamide groups. The appreciable variation in the spleen counts within a group also made interpretation difficult.

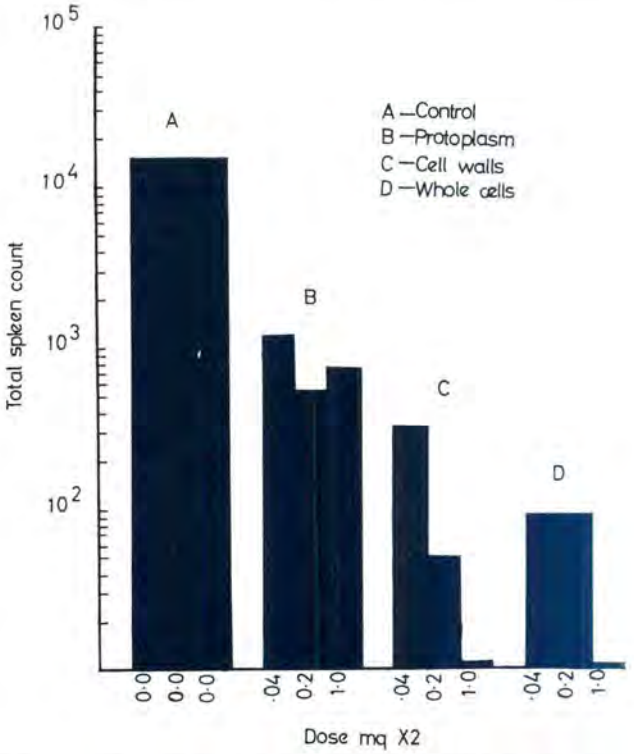

FIG. 1.—Comparison of immunizing properties of whole bacteria, cell walls and protoplasm

In the second experiment, the mice were challenged with 4×10^4 live bacteria and the cumulative deaths recorded in Fig. 2. There was a difference of only 0.13 in the Tangens values between the group which had received formamide extracted cell walls and the control group, while the differences for the ether: ethanol group and the TCA group were 0.44 and 0.35 respectively. The results correspond to those in the first experiment but are more distinct. Ether: ethanol had little effect on the immunizing potency of the cell walls, but extraction with formamide markedly reduced their ability to induce a protective immunological response.

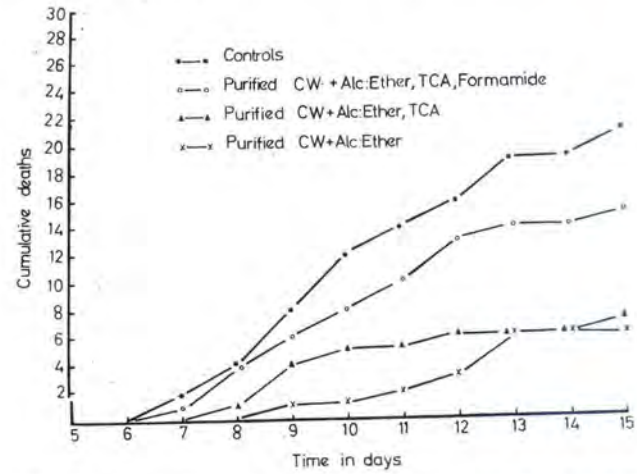

FIG. 2.—Immunizing properties of enzymatically purified cell walls (cw) after successive extraction with ether: ethanol, TCA and formamide. — Mice were challenged with 4×10^4 bacteria

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

TABLE 1. — *Total spleen counts of mice immunized with purified cell walls and subjected to different extraction procedures*
Spleen counts × 10^3 seven days after challenge with 2 × 10^5 living bacteria

Treatment of cell walls		Controls		Ether: Ethanol			Ether: Ethanol and TCA			Ether: Ethanol, TCA & Formamide		
Dose mg × 2		—	—	1.0	0.2	0.04	1.0	0.2	0.04	1.0	0.2	0.04
Mouse No.	1	148.0	480.0	0.62	6.60	44.00	0.10	6.80	0.13	2.00	63.00	6.01
	2	132.0	12.5	5.60	24.00	2.90	3.80	8.40	14.00	1.10	52.30	46.00
	3	180.0	39.0	0.30	14.30	0.64	2.18	62.00	C	1.20	2.50	52.00
	4	46.0	240.0	0.23	C	2.30	56.00	2.50	10.20	4.00	D	C
	5	D	D	1.20	C	C	14.60	0.90	62.00	2.00	D	D
	6	D	D	1.80	0.90	C	2.10	0.20	1.20	12.60	D	D
	7	D	D	0.10	18.30	D	52.30	0.20	16.00	4.80	D	D
	8	D	D	0.70	6.80	D	3.80	0.40	D	0.00	D	D
	9	D	D	1.90	2.50	D	43.90	D	D	D	D	D
	10	D	D	0.40	18.80	D	D	D	D	D	D	D
Mean spleen count of surviving mice		135.0	193.0	1.20	11.50	12.20	19.9	10.2	17.2	3.5	39.3	34.7

C — Plates contaminated D — Died before 7th day

TABLE 2. — *Chemical analysis of cell walls*

Material	Batch No.	Protein	Hexose	Phosphorus	N-acetyl	Pentose
		%	%	%	%	%
Untreated cell walls	1	25.0	13.2	1.4	1.6	—
	2	32.2	19.2	0.25	5.9	16.25
Purified cell walls	1	6.25	15.7	0.045	4.2	24.3
	2	11.0	22.1	0.06	6.6	16.3
Purified cell walls extracted with ether: ethanol	1	8.9	17.8	0.041	5.2	20.6
	2	12.0	22.0	0.06	6.6	11.9
Purified cell walls extracted with ether: ethanol and TCA.	1	9.1	18.7	0.06	7.0	12.3
	2	10.8	22.0	0.06	10.8	21.45
Purified cell walls extracted with ether: ethanol, TCA, and formamide	1	4.43	16.3	0.07	7.7	—
	2	—	—	—	—	—
Ether: Ethanol extract	1	2.0	3.95	0.12	0.2	3.25

Chemical composition of cell walls

The chemical analysis of two batches of cell walls after the different purification and extraction procedures is shown in Table 2.

Although there was some difference between the analysis of the two batches, there was little change in the chemical composition of the cell walls following the successive stages of extraction. The only marked change was a drop in protein content after formamide extraction. The slight discrepancies in the analysis following the extraction procedures and differences between the two batches of cell walls were not due to the techniques used, because repeated analysis of a particular preparation always gave consistent and reproducible results.

Discussion

Antigens capable of inducing a protective immunity have been found to be localized in the cell walls of a variety of bacterial species for example, *Mycobacterium tuberculosis* (Ribi, Larson, Wicht, List & Goode, 1966), *Listeria monocytogenes* (Klasky & Picket, 1968) and *Bordetella pertussis* (Munoz, Ribi & Larson, 1959).

Our results clearly show that the immunizing antigen of *C. pseudotuberculosis* is also present in the cell wall. By applying standard extraction procedures we hoped to obtain some information on the chemical nature of the antigen concerned. Removal of surface lipid by ether: ethanol extraction did not alter the immunizing activity of the walls. Although the lipid contributes to the virulence of the organism (Jolly, 1966), our results indicate that it does not play any role in inducing immunity.

Antibodies to teichoic acids have been shown to be of importance in promoting phagocytosis of staphylococci (Mudd, Yoshida, Li & Lenhart, 1963; Cameron, 1969). This however, does not seem to be true in the case of *C. pseudotuberculosis* because prolonged extraction with TCA did not decrease the immunizing activity of the cell walls. Moreover, there was no significant drop in the phosphorus content of the cell walls and virtually no precipitate could be obtained by alcohol or acetone treatment of the extract. It therefore appears that *C. pseudotuberculosis* walls contain very little teichoic acid.

The immunizing activity of the cell walls was markedly reduced after formamide extraction at 180 °C. However, very little material could be recovered by acetone precipitation of the formamide extract. This suggests that no effective extraction occurred but that denaturation was responsible for loss of biological activity.

The differences in the chemical analysis between the two batches of cell walls tested and the slight discrepancies in the analysis after the successive extraction procedures, are probably due to changes in the solubility of the cell walls and a resultant alteration of their susceptibility to hydrolysis. This would result in a variation in the release of chemically reactive cell wall components. There were, however, no dramatic changes in the composition of the cell walls after the different extraction procedures. This is in accordance with the finding that there is little change in the immunizing properties of the cell walls until the formamide step, and supports the view that the antigen responsible for inducing a protective immunity is an integral part of the cell wall.

Summary

Mice were successfully immunized with cell walls of *C. pseudotuberculosis*. Purification of the cell walls with ribonuclease, trypsin and pepsin and subsequent extraction with ether: ethanol, and TCA did not materially change either their chemical composition or their immunizing potency. However, after extraction with formamide at 180 °C the biological activity was markedly reduced. It is concluded that the protection inducing antigen is an integral part of the cell wall.

Acknowledgements

We wish to express our particular appreciation to Dr. Edgar Ribi and Dr. Jack Munoz of the Rocky Mountain Laboratory, Montana, for their guidance during the early stages of this work.

We wish to thank Mr. H. J. Els for examining the cell wall preparations and preparing the electronmicrograph as well as Miss M. Smit and Mr. A. du Bruyn who drew and photographed the graphs.

Drs. R. D. Bigalke and R. C. Tustin are also thanked for their assistance in compiling the paper.

References

ALLSOP, J. & WORK, E., 1963. Cell walls of *Propionibacterium* species: Fractionation and composition. *Biochem. J.*, 87, 512–519.

AMSTRONG, J. J., BADDILEY, J. & BUCHANAN, J. G., 1960. Structure of the ribitol teichoic acid from the walls of *Bacillus subtilis*. *Biochem. J.*, 76, 610–621.

BLEIWEIS, A. S., KARAKAWA, W. W. & KRAUSE, R. M., 1964. Improved technique for the preparation of streptococcal cell walls. *J. Bact.*, 88, 1198–1199.

CAMERON, C. M., 1969. Antiphagocytic activity of *Staphylococcus aureus* antigens. *Onderstepoort J. vet. Res.*, (this journal).

CAMERON, C. M. & MINNAAR, J. L., 1969. Immunization of mice against *Corynebacterium pseudotuberculosis* infection. *Onderstepoort J. vet. Res.*, (this journal).

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.

CHEN, P. S., TORIBARA, T. Y. & WARNER, H., 1956. Microdetermination of phosphorus. *Analyt. Chem.*, 28, 1756–1758.

HEYMAN, H., MANNIELLO, J. M. & BARKULIS, S. S., 1963. Structure of streptococcal cell walls. I. Methylation study of C-polysaccharide. *J. biol. Chem.*, 238, 502–509.

JOLLY, R. D., 1966. Some observations on surface lipids of virulent and attenuated strains of *Corynebacterium ovis*. *J. appl. Bact.*, 29, 189–196.

KABAT, E. A. & MAYER, M. M., 1964. Experimental Immunochemistry. 2nd Ed. Charles C. Thomas, Springfield, Ill.

KLASKY, S. & PICKETT, J. M., 1968. Immunogenicity and specificity of *Listeria monocytogenes* cell walls. *J. infect. Dis.*, 118, 65–75.

KRAUSE, R. M., 1967. Preparation of cell wall antigens from Gram positive bacteria. *In:* Methods in Immunology and Immunochemistry. Vol. I. Edited by C. A. Williams and M. W. Chase, Academic Press, N. Y. & London.

KRAUSE, R. M. & McCARTY, M., 1961. Studies on the chemical structure of the streptococcal cell wall. I. The identification of a mucopeptide in the cell walls of Group A and A-variant streptococci. *J. exp. Med.*, 114, 127.

KWAPINSKY, J. B., 1965. Methods of Serological Research. John Wiley & Sons, Inc., N.Y., London & Sydney.

MORSE, S. I., 1962. Studies on the chemistry and immunochemistry of cell walls of *Staphylococcus aureus*. *J. exp. Med.*, 116, 229–245.

MUDD, S., YOSHIDA, A., LI, I. W. & LENHART, N. A., 1963. Identification of a somatic antigen of *Staphylococcus aureus* critical for phagocytosis by human blood leucocytes. *Nature, Lond.*, 199, 1200–1201.

MUNOZ, J., RIBI, E. & LARSON, C. L., 1959. Antigens of *Bordetella pertussis*. I. Activities of cell walls and protoplasm. *J. Immun.*, 83, 496–501.

REISSIG, J. L., STROMINGER, J. L. & LELOIR, L. F., 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. *J. biol. Chem.*, 217, 959–966.

RIBI, E. & HOYER, B. H., 1960. Purification of Q-fever rickettsiae by density gradient sedimentation. *J. Immun.*, 83, 314–318.

RIBI, E., LARSON, C., WICHT, W., LIST, R. & GOODE, G., 1966. Effective non-living vaccine against experimental tuberculosis in mice. *J. Bact.*, 91, 975-983.