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Properties of an Antigenic Polysaccharide from *Corynebacterium parvum*

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Corynebacterium parvum strain 10390 is an antitumor agent and stimulant of the reticuloendothelial system and produces a soluble antigen towards the end of its growth cycle. This material, which is a cell wall component and can also be released from the organism by acid or alkaline hydrolysis, has been purified. It is an acidic polysaccharide of molecular weight 100,000 to 150,000 and contains galactose, glucose, fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, uronic acids, sialic acids, and a small proportion of amino acids. The antigen gives a precipitin reaction with antisera raised against the whole organism and also binds to animal cells. The antigenic determinants are extremely resistant to oxidation, reduction, and enzymatic and chemical hydrolysis, but the single cell-binding site is destroyed by alkali and also by *Helix pomatia* digestive juice, alginase, and neuraminidase without substantially affecting the molecular weight. This site is inaccessible until the molecule is released from the cell surface. The possibility that the soluble antigen is the biologically active fraction of *C. parvum* is discussed.

Certain anaerobic coryneforms, in particular *Corynebacterium parvum*, have recently been intensively investigated as antitumor agents (22, 23) and stimulants of the mononuclear phagocyte system (6, 14, 21). These properties appear to be associated with the cell wall (15).

Recently, the anaerobic coryneforms have been divided into four groups on the basis of cell wall composition, deoxyribonucleic acid homology, and serological relationships (8). However, investigations into the biological effects of various strains have indicated that activity is not restricted to any one group of organisms (14, 21) but may be a property of those strains that are antigenically closely related and release soluble, cross-reacting material from the cell wall (W. H. McBride, J. Dawes, N. Dunbar, A. Ghaffar, and M. F. A. Woodruff, Immunology, in press). The production, quantitation, and purification of this antigen, as well as some of its physical, chemical, and immunological properties, are described here.

MATERIALS AND METHODS

Organism. *C. parvum* 10390 was obtained from the National Collection of Type Cultures, Colindale, England. Unless otherwise stated, cultures were grown in beef digest broth plus 3% glucose at 37 C. The broth was prepared as follows. One kilogram of defatted minced steak was steamed in 7 liters of water. Anhydrous Na_2CO_3 (25 g) was added, the pH was adjusted to 8.0 with 1 N NaOH, and the mixture was incubated with 35 g of pancreatin at 60 C for 6 h. Concentrated HCl (40 ml) was added, and after steaming for 1 h the digest was left overnight at 4 C. The supernatant was filtered, 70 g of proteose peptone (Difco) was added to the filtrate, the pH was adjusted to 8.0 with 10 N NaOH, and 8.78 g of $CaCl_2$ was added. The solution was steamed, filtered, and finally adjusted to pH 7.4 with 1 N HCl and autoclaved. Sterile 20% glucose solution was added to a final concentration of 3%. Bacteria were grown in sealed bottles without shaking; incubation under completely anaerobic conditions was unnecessary. Cultures were harvested in late stationary phase, and the supernatant was used as source of soluble antigen. Bacteria were washed in 0.9% NaCl, killed by exposure to 0.5% formalin for 24 h at 4 C, washed four times to remove formalin, and resuspended in 0.9% NaCl at a final concentration of 7 mg/ml (dry weight). This corresponds to approximately 3×10^{11} organisms/ml.

Viable counts were measured on blood agar after incubation in an atmosphere of 90% H_2-10% CO_2 for 3 days.

Strains could be stored for up to 3 months at 4 C in stabs of beef digest broth plus 3% glucose, solidified with 1.5% agar. Samples were lyophilized for longer storage.

Assay of soluble antigen. The soluble antigen was routinely detected and assayed by counterimmunoelectroosmophoresis (CIEOP) in 1% agarose against rabbit antisera. The method used was that of Prince and Burke (16), but using Veronal buffer (pH 8.2, ionic strength 0.033). For quantitative estimation,

doubling dilutions of each sample were tested against rabbit antiserum to *C. parvum* strain 10390.

The precipitin reaction of antigen with rabbit antiserum against *C. parvum* 10390 was carried out essentially as described by Morse (13). Different quantities of antigen (2 to 50 μg) were added to 50 μliters of antiserum in a final volume of 1 ml of 0.9% saline, and incubated at 37 C for 2 h. After 24 h at 4 C the precipitate was removed by centrifugation, washed twice, and dissolved in 0.5 ml of 1 N NaOH before the protein concentration was measured by the method of Layne (9).

Ability of antigen to bind to sheep erythrocytes was assayed by the passive hemagglutination technique described by McBride et al. (12).

Isoelectric focusing. Isoelectric focusing was carried out in polyacrylamide gels (24). Ampholyte gradients of pH 3 to 10 and pH 4 to 6 were used at gel concentrations of 7.5 and 3.75%. Samples were focused for 16 h. The gels were then embedded in 1% agarose in Veronal buffer, and CIEOP at right angles to the polyacrylamide gel was used to detect the presence of antigen.

Protein estimation. Soluble protein was routinely assayed by the spectrophotometric method of Layne (9). Protein concentrations less than 0.1 mg/ml were measured by the method of Lowry et al. (11).

Analytical methods: amino acid analysis. Samples were hydrolyzed in 6 N HCl at 100 C for 24 h and analyzed on a Locarte (London) automatic amino acid analyzer.

Monosaccharide analysis. After hydrolysis of the sample with 0.3, 0.5, or 2.0 N HCl at 100 C for 16 h, a range which covers optimum hydrolytic conditions for neutral and amino sugars, the constituent monosaccharides were converted to their trimethylsilyl ethers and analyzed by gas-liquid chromatography as described by Schrager and Oates (17).

Uronic acid content. The uronic acid content of the antigen was estimated by using a modification of Dische's original method (7).

Sialic acid estimation. The sample was incubated in acetate buffer (pH 5.1) at 37 C for 2 h with a dialyzed culture filtrate of *Clostridium perfringens*. This contains several glycosidases (10), including a neuraminidase (EC 3.2.1.18) specific for sialic acids with $2 \rightarrow 3$ or $2 \rightarrow 6$ α-ketosidic linkages; it is not known to release other 2-keto-3-deoxy-sugar acids. Alternatively the sample was heated at 80 C for 2 h in 0.01 N H_2SO_4. Both methods released the same amounts of sialic acid, which was assayed by the thiobarbituric acid method of Aminoff (1) and expressed in terms of an *N*-acetyl neuraminic acid standard.

Phosphorus content. The total phosphate content of a sample was measured by the method of Chen et al. (2).

Treatment with enzymes. Antigen was incubated with enzymes at 37 C for 24 h unless otherwise stated.

Proteases. Samples were incubated with trypsin (EC 3.4.4.4), pepsin (EC 3.4.4.1), and Pronase at an enzyme concentration of 100 μg/ml in 0.15 M phosphate buffer (pH 7.2).

Carbohydrases. Lysozyme (EC 3.2.1.17) was used at a concentration of 100 μg/ml in 0.15 M phosphate buffer (pH 7.2). Testicular hyaluronidase (EC 3.2.1.d) and bee venom containing a hyaluronidase of the β-*N*-acetyl-glucosaminidase type (EC 3.2.1.e) were used at the same concentration but in 0.4 M acetate buffer (pH 5.0). *Helix pomatia* digestive juice, a source of β-glucuronidase (EC 3.2.1.31), and a bacterial alginase (EC 3.2.1.16) that hydrolyzes ester linkages involving guluronic acid were also used in 0.4 M acetate buffer (pH 5.0) at final dilutions of 1:10. Treatment with *C. perfringens* neuraminidase is described above.

Phospholipase A. Phospholipase A (EC 3.1.1.4) was incubated with the antigen at a dilution of 1:10 in 0.15 M phosphate buffer (pH 8.0).

Enzyme sources. Pronase was obtained from Calbiochem, San Diego, Calif., bee venom grade II from Sigma Chemical Co., St. Louis, Mo., and trypsin, pepsin, lysozyme, and testicular hyaluronidase from BDH Chemicals Ltd., Poole, England. *H. pomatia* digestive juice was from Koch-Light Laboratories, Colnbrook, England, and bacterial alginase was a gift of I. W. Davidson and I. W. Sutherland, Department of Microbiology, University of Edinburgh. Phospholipase A was a gift of P. C. Wilkinson, Department of Bacteriology and Immunology, University of Glasgow.

Treatment with chemicals: hydrolysis. Samples were hydrolyzed with 0.25 N NaOH or 0.25 N HCl at 60 C for times up to 24 h.

Oxidation. Antigen was incubated with 0.1 M sodium metaperiodate in 0.2 M acetate buffer (pH 5.8) in the dark at room temperature for times up to 72 h.

Reduction. A sample was incubated with 0.05 M $NaBH_4$ in 0.4 M acetate buffer (pH 5.0) for 1 h at room temperature.

RESULTS

Occurrence of soluble antigen. Strains of anaerobic coryneforms classed by Johnson and Cummins (8) in *Corynebacterium acnes* groups I and II and *Corynebacterium avidum* group IV characteristically produce a soluble, antigenically cross-reacting material derived from the cell wall (McBride et al., Immunology, in press). Since *C. parvum* strain 10390 produces large amounts of the antigen, it was routinely used as a source, and this report is concerned with the characteristics of the antigen produced by this particular strain. Although the organism was usually grown in beef digest broth plus 3% glucose, it also grew in the medium without glucose and in cooked meat broth. Soluble antigen was produced in all three media.

The material was detected by techniques that rely on its antigenicity (see Materials and Methods). Titration of the antigen in CIEOP indicated that this method will detect 40 ng of material. No other satisfactory way has yet been found to quantitate the antigen.

The time course of production of soluble

antigen throughout the growth curve of the organism, detected by its antigenicity on CIEOP, is shown in Fig. 1. No soluble antigen was released during logarithmic growth, but its concentration subsequently rose rapidly to a $\log_2$ titer of 9. This rise appeared to be associated with death, but not lysis, of the bacteria, since although the turbidity did not decrease, the stationary phase was very short and the viability dropped rapidly.

Antigen with all the characteristics of that detected in the growth medium was released in considerable amounts from formalin-killed, washed organisms by treatment with 0.25 N HCl at 60 C for 1 h. This release, like that at the end of the growth cycle, occurred without a decrease in turbidity, although it was associated with the appearance of protein in the supernatant (Fig. 2). Alkaline hydrolysis (0.25 N NaOH; 60 C for 1 h) gave a similar antigen except that it had lost its ability to bind to erythrocytes (see Table 2). Moreover, hydrolytic release of antigen by alkali was associated with a decrease in optical density (540 nm) of the suspension as well as release of protein, and is presumably associated with loss of cellular integrity. The antigen was not released by incubation of whole organisms with lysozyme, Pronase, neuraminidase, snail digestive juice, or bacterial alginase, but it could be detected in the supernatant from formalin-killed organisms stored in saline at 4 C for several weeks.

Purification of soluble antigen. Growth medium containing antigen was treated with Pronase (100 μg/ml; 37 C for 2 h) to hydrolyze the protein components of the medium. Ethanol was added to 70% and the precipitated antigen was removed by centrifugation and redissolved in 1/100 the original volume of 0.9% NaCl. These steps removed 98% of the contaminating protein with only 5% loss of antigen. Pronase treatment was omitted when material for chemical analysis was required. Further purification on Sephadex G-200, using 0.9% NaCl as eluant, gave a single antigen peak almost entirely free of detectable protein (Fig. 3). Correlation of the amount of material obtained with the theoretical sensitivity of CIEOP indicated that antigen comprises most of this fraction. Purified antigen was stored at −20 C or freeze-dried.

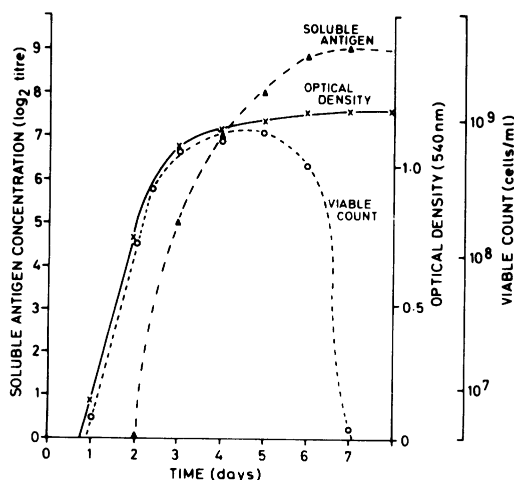

FIG. 1. *Release of soluble antigen during the growth cycle of C. parvum. After measurement of optical density (540 nm) and plating for viable count, culture samples were centrifuged and antigen concentration in the supernatant was determined by counterimmunoelectroosmosphoresis.*

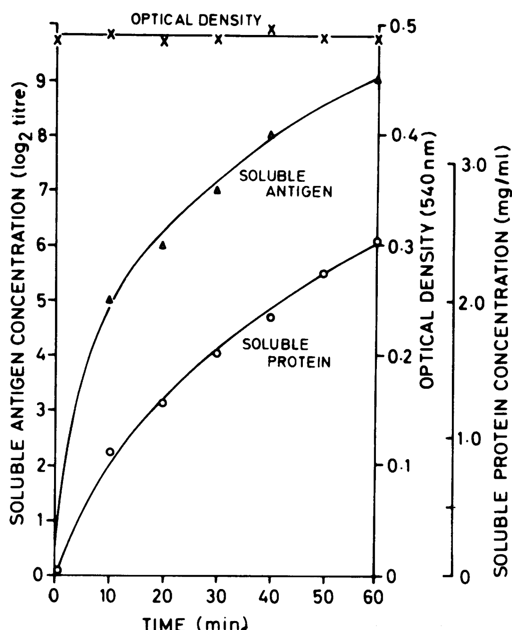

FIG. 2. *Release of soluble antigen by acid hydrolysis of C. parvum. A suspension of C. parvum (6 mg [dry wt]/ml) was hydrolyzed with 0.25 N HCl at 60 C. The optical density (540 nm) of a sample was measured; it was then centrifuged and the supernatant was assayed for soluble protein and soluble antigen.*

Physical properties of the antigen. The antigen was nondialyzable and fully retained by Amicon Centriflo membrane ultrafilters with a molecular weight cut-off of approximately 50,000. Using blue dextran 2000 to mark the void volume, the antigen was found to be excluded from Sephadex G-25, G-50, and G-100 but was retarded in G-200, from which it eluted as a single peak. The molecular weight of the antigen is therefore greater than 100,000, and on

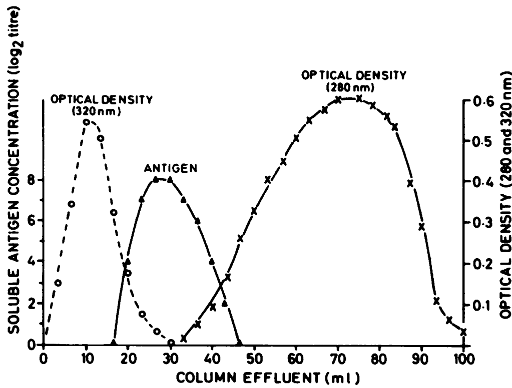

FIG. 3. *Chromatography of C. parvum soluble antigen on Sephadex G-200. Supernatant from cultures of C. parvum was dialyzed against 0.9% NaCl and vacuum-concentrated. Blue dextran 2000 was added as solvent front marker and detected by its absorbance at 320 nm. Absorbance at 280 nm indicated the presence of protein in other fractions. In a separate run without blue dextran, protein was absent from the first 30 ml of effluent. Antigen concentration was determined by counterimmunoelectroosmosphoresis.*

the basis of comparison with dextran standards probably lies in the range 100,000 to 150,000.

The antigen was highly negatively charged, with the isoelectric point, as determined by focusing in acrylamide gel, being in the range 3.5 to 4.0.

It had no absorption peak in the wavelength range 260 to 450 nm.

Chemical composition of the antigen. Results of chemical analyses of the purified antigen are shown in Table 1. The molecule was largely carbohydrate, the major sugar components being galactose and *N*-acetylglucosamine. Glucose, fucose, *N*-acetylgalactosamine, and the uronic and sialic acids were also present, but there was only a trace of mannose. Rhamnose and arabinose were absent. Amino acids constituted less than 10% of the antigen, and there were no dicarboxylic or aromatic residues. Phosphate was present in trace amounts. These assays account for approximately 60% of the weighed sample, and it is likely that the conditions used to release monomeric amino acids and sugars for analysis destroyed less stable constituents that have yet to be recognized. The yield of those detected may also have been reduced. The discrepancy is unlikely to be due to lipid, since the antigen was highly miscible in water, partitioned with water in a chloroform-methanol-water (5:10:4, vol/vol) mixture, and did not stain with Sudan black.

Biological properties of the antigen: reaction with antibody. Although the antigen did not appear to be immunogenic, it reacted with antibody prepared against the whole organism. The tube precipitin reaction yielded a curve characteristic of that obtained with many polysaccharide antigens (Fig. 4). The antigen could also be readily detected by precipitation with antisera in immunodiffusion or CIEOP, and normally gave one major and two minor lines. To identify the antigenic determinants, attempts were made to destroy them chemically, physically, and enzymatically (Table 2). Enzymatic treatment with proteases, carbohydrases, lysozyme, neuraminidase, and phos-

TABLE 1. *Carbohydrate and amino acid composition of C. parvum soluble antigen*

Substance	Amt (μmol/mg)
Carbohydrate	
Galactose	0.52
Glucose	0.32
Mannose	Trace
Fucose	0.23
N-acetylgalactosamine	0.19
N-acetylglucosamine	0.43
Sialic acids	0.20
Uronic acids	0.23
Amino acid	
Alanine	0.060
Arginine	0.014
Aspartic acid	0.035
Glutamic acid	0.078
Glycine	0.066
Histidine	0.021
Isoleucine	0.017
Leucine	0.027
Lysine	0.024
Methionine	0.009
Proline	0.088
Serine	0.102
Threonine	0.119
Valine	0.039

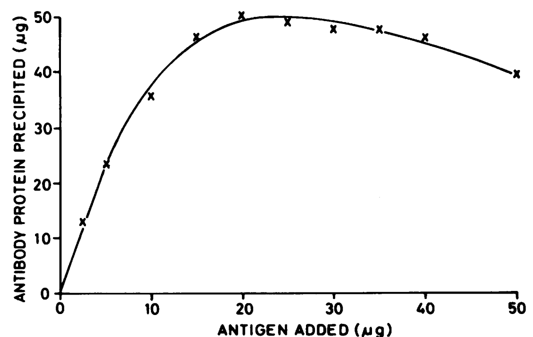

FIG. 4. *Precipitin curve of C. parvum soluble antigen and rabbit antibody. Antibody was raised against formalin-killed preparations of the whole organism.*

TABLE 2. *Sensitivity of C. parvum soluble antigen to physical, chemical, and enzymatic attack*

Treatment	Reagent	Conditions	Sensitivity[a]	
			Antigenicity	Cell-binding capacity
Physical		100 C, 3 h	–	–
Chemical	0.25 N HCl	60 C, 1 h	–	–
		60 C, 24 h	+	Cannot be tested[b]
	0.25 N NaOH	60 C, 1 h	–	+
		60 C, 24 h	+	Cannot be tested[b]
	0.1 M $NaIO_4$	pH 5.8, 20 C, 24 h	–	–
		pH 5.8, 20 C, 72 h	+	Cannot be tested[b]
	0.5 M $NaBH_4$	pH 5.0, 20 C, 1 h	–	–
Enzymatic	Trypsin, pepsin, Pronase	100 μg/ml, pH 7.2, 37 C, 24 h	–	–
	Lysozyme	100 μg/ml, pH 7.2, 37 C, 24 h	–	–
	Hyaluronidases	100 μg/ml, pH 5.0, 37 C, 24 h	–	–
	Helix pomatia digestive juice	0.1 ml/ml, pH 5.0, 37 C, 24 h	–	+
	Alginase	0.1 ml/ml, pH 5.0, 37 C, 24 h	–	+
	Neuraminidase	0.1 ml/ml, pH 5.0, 37 C, 24 h	–	+
	Phospholipase A	0.1 ml/ml, pH 9.0, 37 C, 24 h	–	–

[a] + indicates destruction of property.
[b] Note that cell-binding capacity cannot be measured if antigenicity has been destroyed.

pholipase A left the antigenic moiety intact. Moreover, it was not destroyed by heating at 100 C for 3 h, by hydrolysis in 0.25 N NaOH or HCl at 60 C for 2 h, or by reduction with $NaBH_4$. The antigen was also relatively resistant to periodate oxidation, although treatment with 0.1 M sodium metaperiodate at pH 5.8 destroyed it slowly over a 72-h period, as did prolonged hydrolysis with either acid or alkali.

Binding to red cells. The soluble antigen bound to human, mouse, and sheep red cells, as well as to mouse fibrosarcoma cells. It could be detected by specific agglutination of the coated cells with antibody to *C. parvum* 10390. In the absence of antibody there was no autoagglutination, which indicates that each molecule may contain only one binding site. This site was distinct from and considerably more labile than the antigenic determinants. Binding but not antigenicity was destroyed by alkaline hydrolysis (0.25 N NaOH; 60 C for 2 h), snail digestive juice, bacterial alginase, and *Clostridium perfringens* neuraminidase. Neither was affected by mild acid hydrolysis (0.25 N HCl; 60 C for 2 h), proteases, lysozyme, phospholipase A, and hyaluronidases. Those conditions that destroyed binding to red cells did not grossly affect the molecular weight of the antigen as judged by the elution profiles on Sephadex G-100 and G-200.

Although the antigen was a component of the bacterial cell wall, the whole organism did not bind to red cells. It therefore seemed likely that the binding site of the antigen is inaccessible when it is still attached to the bacterial surface. To test this hypothesis, a suspension of formalin-killed, washed *C. parvum* 10390 was incubated with alginase for 24 h at 37 C, and after centrifuging, washing, and resuspending, the bacteria were treated with 0.25 N HCl at 60 C for 1 h to release the antigen from the bacterial wall. This antigen preparation attached to red cells, but after a further alginase treatment it no longer did so. Alginase, therefore, does not act on the antigen when it is still an integral part of the bacterial surface. Attempts to release sialic acids from the whole organism were also negative, indicating that the neuraminidase substrate is also inaccessible under these circumstances.

DISCUSSION

The *C. parvum* soluble antigen described here is released into the growth medium at the end of logarithmic phase, probably coincident with cell death. Release is not, however, accompanied by lysis of the cell. Antigen is also found in saline in which formalin-killed organisms have been stored, and can be dissociated from whole organisms by mild hydrolysis with or without loss of the integrity of the cell. It seems likely that the molecule is not an extracellular

product but rather an integral component of the bacterial wall, and its release at the end of the growth cycle is probably the result of partial autolysis. The mode of attachment of the molecule to the cell envelope was not determined. If covalent it is either inaccessible or unsusceptible to cleavage by neuraminidase, β-glucuronidase, guluronidase, or *N*-acetylglucosaminidase. The overall structure of the cell wall may resemble that of the plant pathogens *C. poinsettiae* and *C. betae*, in which acidic polysaccharides are covalently linked to peptidoglycan, although hydrolysis destroys the linkage and releases polysaccharide essentially free from muramic acid (5).

The antigen is a high-molecular-weight acidic polysaccharide, linked to a peptide chain or chains which make up less than 10% of the molecule. The major sugars are galactose and *N*-acetylglucosamine, but the material is a complex polysaccharide that also contains considerable amounts of glucose, fucose, *N*-acetylgalactosamine, uronic acids, and sialic acids. The sensitivity of the red cell binding site to alginase indicates that the uronic acids probably include guluronic acid. Lipid is not a major component of the molecule, and the absence of diaminopimelic acid and the low phosphate content exclude the possibilities that mucopeptide or teichoic acid may contribute substantially. Only 60% of the measured weight of the antigen has so far been accounted for, and further chemical studies are now being undertaken to identify other components.

The sugar composition of coryneform cell walls has been used as a major taxonomic criterion (4). The anaerobic commensals contain neither arabinose, the characteristic sugar of classical animal pathogenic corynebacteria, nor rhamnose, which is found in the cell walls of some pathogenic corynebacteria as well as classical propionibacteria (3, 4). The analysis of soluble antigen from *C. parvum* 10390 is in agreement with these findings. However, Johnson and Cummins (8) subdivided the anaerobic coryneforms into four groups, partly on the basis of their cell wall composition. They did not detect the fucose which we found in *C. parvum* 10390 and which Werner and Mann (19) found in other *C. acnes* group II strains, nor did they find any galactose in *C. parvum* 10390. The absence of galactose was used as a criterion for inclusion of a strain in *C. acnes* group II. It should be emphasized that their technology favors loss of the soluble antigen during purification of the cell wall, and this could account for the discrepancy.

The resistance of the soluble antigen to chemical and enzymatic destruction has thwarted efforts to identify the antigenic determinants by this approach. Currently, cross-reactivity with antisera prepared against defined antigens and inhibition of the precipitin reaction by monosaccharides are being investigated in further attempts to solve this particular problem.

More is known of the single site on the molecule which binds to animal cells. It is destroyed by mild alkaline hydrolysis, neuraminidase, β-glucuronidase, and guluronidase, but unaffected by acid hydrolysis, proteases, lysozyme, hyaluronidases, and phospholipase A, and its destruction does not grossly reduce the molecular weight of the molecule. These results are consistent with its involving a short sequence of uronic acid units. The binding site is inaccessible when the antigen is still part of the bacterial cell wall, and it may represent the site of normal attachment to the bacterium.

Extracellular and surface polysaccharides of a wide range of microorganisms have biological activity in animals. Acid polysaccharides from *Escherichia coli* are pyrogenic for rabbits and toxic to mice (20). Moreover, mild alkaline hydrolysis, which destroys the cell binding capacity of the *C. parvum* soluble antigen, also inactivates the *E. coli* acid polysaccharide. The report that purified acid polysaccharides from *Serratia marcescens* have a pronounced antitumor activity (18) is of particular interest in relation to the biological effects of *C. parvum* on tumor growth and the reticuloendothelial system, and we are currently investigating the possibility that it is the *C. parvum* soluble antigen, also an acid polysaccharide, that is the active fraction of this organism.

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