The Relationship of Detergent-Solubilized Plasma-Membrane Components of Rabbit Alveolar Macrophages to an Isolated Inhibitor of Phagocytosis

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1. A plasma-membrane fraction prepared from rabbit alveolar macrophages by hypo-osmotic borate lysis is described. 2. Rabbit lung lavages, containing a glycoprotein inhibitor of phagocytosis, may be fractionated by preparative isoelectric focusing in the presence of Triton X-100. 3. Chemical analysis indicates that the glycoproteins of the lung lavage contain sialic acid, fucose, mannose, galactose, hexosamine and appreciable quantities of glucose. 4. The relationship of macrophage membrane glycoproteins, solubilized with Triton X-100 in the presence of borate, to the lung lavage glycoproteins is demonstrated immunoelectrophoretically.

The diversity of oligosaccharide structure possible within complex carbohydrates might suggest that the saccharide residues present in membrane glycoproteins have a role in molecular recognition at the cell periphery. Involvement of surface sugars in recognition has been inferred from experiments in which lectins inhibited the endocytosis of particles by phagocytes (Allen *et al.*, 1971; Berlin, 1972; Brown *et al.*, 1975; Edelson & Cohn, 1974; Friend *et al.*, 1975; Goldman, 1974*a,b*; Goldman & Cooper, 1975; Lutton, 1973; Raz & Goldman, 1976). In addition, inhibitors of phagocytosis have been obtained from rabbit lung lavages (Ulrich & Zilversmit, 1970) and peritoneal granuloma of the guinea pig (Bole *et al.*, 1975; Bole & Wright, 1976). These preparations from both animals contained carbohydrate and protein, but were low in lipids. It was suggested that some components of the rabbit lung lavage inhibitor were derived from the surface of the alveolar macrophage (Ulrich & Zilversmit, 1970).

By using human erythrocyte stroma as a model, we have found that the reversible binding of borate to *cis*-vicinal diols promotes the solubilization by Triton X-100 of glycoproteins low in sialic acid [see the preceding paper (Pratt & Cook, 1979)]. The utilization of this finding has enabled us to investigate the relationship of the inhibitor of phagocytosis described by Ulrich & Zilversmit (1970) to the glycoproteins of the macrophage plasma membrane. We consider that the present study provides a further step towards the understanding of the biological role of these macromolecules at the cell periphery.

Materials and Methods

Isolation of rabbit alveolar macrophages

Alveolar macrophages were obtained from New Zealand White rabbits of either sex. The procedure of Leake & Myrvik (1968) was used, in which the lungs of killed animals were aspirated with phosphate-buffered saline (Cook & Eylar, 1965) on the eighth day after the intravenous administration of heat-killed freeze-dried BCG (Bacillus Calmette–Guerin) (5 mg/rabbit) kindly provided by the BCG Unit, Glaxo Laboratories Ltd., Greenford, Middx., U.K., in an emulsion of sterile saline (0.9%, w/v, NaCl) and Marcol 52 oil (1:1, v/v). We found it easier to remove the lungs from the rabbit within 5 min of death. The lungs were canulated via the trachea by using a stainless-steel canula through which aspiration was effected. During aspiration the lungs were covered with surgical gauze soaked in phosphate-buffered saline warmed to 37°C. After filling the lungs with saline they were kneaded gently by hand, this being followed by aspiration via the stainless-steel canula. Aspirates were cooled on ice and centrifuged for 4 min at 4°C and 500*g* (*r*_{av} 11.0 cm). The supernatant fluid was carefully removed for further processing (see under 'Preparation of phagocytosis inhibitor and lung lavage fractions') and the cell pellets were washed three times in at least 15 vol. of either Krebs–Ringer phosphate buffer or borate-buffered saline (Warley & Cook, 1973). Cell preparations were checked for viability by the exclusion of Erythrosin B as described by Phillips & Terryberry (1957); routinely we found that the viability of the washed cell preparations was in excess of 95%. Purity of cell preparations was checked microscopically on smears stained with May–Grunwald and Giemsa stains and found to consist of 98% macrophages, the remainder

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being lymphocytes and erythrocytes. Ciliated cells from the tracheal mucosa were absent.

Preparation of rabbit alveolar macrophage plasma membranes

Plasma membranes were isolated from suspensions of washed macrophages, in 0.15M-NaCl made 1.0M with respect to CaCl_2 and MgCl_2 and containing 50mM-borate, pH7.2, by using the borate extraction technique of Warley & Cook (1973). Membranes were purified on a column of Superbrite 150 glass beads (kindly provided by 3M Company, Penge, London SE20 7TR, U.K.) as described by the above authors. The filtrate obtained directly from the column was designated 'post-column fraction I', and the column then eluted with distilled water (15ml). In the latter case the first 5ml of eluate is termed 'fraction II' and the remaining material 'fraction III'.

Solubilization of macrophage membranes by Triton X-100

Samples of macrophage plasma membranes were resuspended in distilled water adjusted to pH8.6 with 1M-NaOH. Portions of this suspension were centrifuged for 1 h at 4°C and 100000g (r_{av} , 9.86cm) and the membrane pellets resuspended either in distilled water (adjusted to pH8.6) or 50mM-borate, pH8.6, and kept at 4°C for 1 h. After this period the suspensions were made 1% (v/v) with respect to Triton X-100; stock solutions of either 10% (v/v) Triton X-100, pH8.6, or 10% (v/v) Triton-100 containing 50mM-borate, pH8.6, were used. After a further period of 30 min at 4°C the extracts (concentration of protein 0.5–1.0mg/ml) were centrifuged for 1 h at 4°C at 100000g. The supernatant fluid was considered to contain solubilized membrane constituents.

Preparation of phagocytosis inhibitor and lung lavage fractions

The starting material was the supernatant fluid of the lung lavage from which the cells had been removed by centrifugation. Routinely the lavages were centrifuged for 20 min at 4°C and 20000g (r_{av} , 9.86cm) to remove any cell debris. An inhibitor of phagocytosis was prepared from this fluid by using the method described in detail by Ulrich & Zilversmit (1970), in which inhibitory material remains in solution after treatment of the lavage with ice-cold HClO_4 . Freeze-dried inhibitor was stored desiccated at -20°C.

In order to control for the possibility that the inhibitor might be contaminated with membrane fragments, a sample of lung lavage was centrifuged for 1 h at 4°C at 100000g (r_{av} , 9.86cm), and the supernatant fluid dialysed extensively against distilled

water and freeze-dried. This material was used as a control when examining the nature of the phagocytosis inhibitor.

The same lung material (320mg of protein) was fractionated in 1% (v/v) Triton X-100 by preparative-column isoelectric focusing on a 5–50% (w/v) linear sucrose gradient containing 1% (w/v) carrier ampholytes (pH2.5–6.0) by using an LKB 8102 column of 440ml capacity (LKB-Produkter A.B., Stockholm, Sweden). The apparatus was operated at 4°C at a maximum power of 6W for up to 36 h when a steady minimum current had been established for 2 h. Fractionated material was collected from the base of the column, the pH gradient being monitored with a flow electrode (Activion type 003-11-306). To control for the possible contamination of the dialysed fractions by traces of sucrose, a further quantity of lung material (93mg of protein) was subjected to preparative isoelectric focusing in 1% (v/v) Triton X-100 on a linear 0–75% (v/v) ethanediol (AnalaR grade; BDH Chemicals Ltd., Poole, Dorset, U.K.) gradient containing 1% (w/v) carrier ampholytes (pH3.5–10.0).

Assay of phagocytosis by using latex beads

Uptake of polystyrene latex particles by rabbit alveolar macrophages over a 2 h period at 0 and 37°C was determined by the methods described in detail by Ulrich & Zilversmit (1970). Dow latex (polystyrene) beads of 0.822 μm diameter were obtained from Serva Fine Biochemicals, Heidelberg, Germany. Latex beads were administered to the cells in the presence of Tween 80 [purchased from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K., and used at a final concentration of 0.001%, v/v] to prevent clumping of the latex particles (Ulrich & Zilversmit, 1970), and the reaction was terminated by the addition of 1.3mM-sodium iodoacetate and 0.02mM-dicoumarol (final concentrations). The polystyrene latex was measured by extraction into dioxan (4.0ml) at room temperature for 24 h and measuring the A_{253} of the extract. The absorbance measurements were corrected for blank determinations on the same number of macrophages incubated without latex beads.

Preparation of antisera

Guinea pigs received three intramuscular injections (0.5ml) at 2-week intervals of dialysed and freeze-dried lavage (20mg/ml) in a 1:1 (v/v) emulsion of sterile 0.9% (w/v) NaCl and Freund's complete adjuvant. At 2 weeks after the last inoculation, the animals were anaesthetized with diethyl ether and blood was removed by cardiac puncture. The presence of antibodies in the sera was checked by a positive reaction in an interfacial ring test obtained by overlaying the sera with untreated freeze-dried

lavage dissolved in phosphate-buffered saline (20 mg/ml). Preinoculation sera from all the animals were tested, and a negative precipitin reaction was found in all cases. We are grateful to Dr. D. Morton of this laboratory for performing all the operations involving the use of the live guinea pigs.

Electron microscopy

Samples of pelleted material were processed for electron microscopy as detailed elsewhere (Warley & Cook, 1976), and were examined in a GEC AE1 EM6B electron microscope. Care was taken to examine all regions of the pellet thoroughly.

Immunoelectrophoresis and analytical isoelectric focusing

Immunoelectrophoresis was performed in a commercial apparatus (Paynes and Byrne Ltd., Greenford, Middx., U.K.). 'Rocket' and 'diffused rocket' immunoelectrophoresis was carried out on 10 cm × 10 cm or 5 cm × 5 cm glass plates with an applied potential of 3V/cm for a period of 18 h. Two-dimensional or crossed immunoelectrophoresis was performed by the procedure of Clarke & Freeman (1968).

Analytical isoelectric focusing was performed in thin layers of polyacrylamide gel over a pH gradient 3.5–10.0 by using a Multiphor apparatus (LKB-Produkter A.B.). Preparation of the samples and the gels used in the present work has been described in detail elsewhere (Cook, 1976).

Polyacrylamide-gel electrophoresis

Electrophoresis was performed in the presence of sodium dodecyl sulphate in 10% (w/v) acrylamide gels as described by Fairbanks *et al.* (1971). Gels were stained with Coomassie Brilliant Blue (BDH) or with the periodic acid/Schiff technique by using the procedure described by Fairbanks *et al.* (1971).

Assay of enzymic activities

5'-Nucleotidase (EC 3.1.3.5) was determined by using the procedure described by Persijn *et al.* (1968). Phosphodiesterase I (EC 3.1.4.1) was measured by the method of Touster *et al.* (1970), with *p*-nitrophenyl 5'-thymidylate (Sigma) as substrate; non-specific diesterase activity was controlled for by substitution of bis-(*p*-nitrophenyl) phosphate for the substrate, and care was taken to check for non-enzymic hydrolysis of the substrates. Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of Pennington (1961) as modified by Porteous & Clark (1965), and NADH oxidoreductase (EC 1.6.4.3) by the method of Wallach & Kamat (1964). Acid phosphatase (EC 3.1.3.2) was determined as a function of the release of *p*-nitrophenol from disodium *p*-nitrophenyl orthophosphate (Boehringer Mannheim G.m.b.H., Marburg, Germany), and

glucose 6-phosphatase was examined by the method of Hübscher & West (1965). All determinations were performed in duplicate, and, where sufficient material was available, in triplicate. Where quoted in the Results section, values are given as means ± s.d.

Chemical analyses

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin prepared as a protein standard by Armour Pharmaceutical Co., Eastbourne, East Sussex, U.K. Inorganic phosphorus was measured by means of the method of Fiske & SubbaRow (1925), samples containing organic phosphorus being digested at 180°C for 2 h in 72% (w/v) HClO₄ before analysis.

Sialic acid was determined by the 2-thiobarbituric acid method of Warren (1959), which measures hydrolysis in 0.05 M-H₂SO₄ at 80°C for 1 h, with *N*-acetylneuraminic acid (Sigma type IV) as a standard. Hexosamine was determined on a JEOL JLC-5AM amino acid analyser, with D-glucosamine hydrochloride (Sigma) as a standard; samples were flushed with N₂ and hydrolysed under vacuum in 4M-methanesulphonic acid (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) at 100°C for 20 h. Standard solutions of hexosamine were found to give the following yields: D-glucosamine (82%) and D-galactosamine (94%), after the same acid treatment and chromatography conditions used with the test samples.

Glucose was determined colorimetrically by using the combined glucose oxidase/peroxidase procedure essentially as described by Raabo & Terkildsen (1960). The materials for this procedure were purchased from Sigma; samples were hydrolysed in 1M-H₂SO₄, and a neutral sugar fraction was prepared before analysis by ion-exchange chromatography as described by Spiro (1966).

Analysis by g.l.c.

Lung lavage material was analysed for sugars by g.l.c. by using the procedure of Chambers & Clamp (1971). A Pye series 104 chromatograph was used with a 255 cm (diameter 0.4 cm) column containing 3% silicone gum rubber SE-30 on highly purified Chrom W-AW-DMCS 80/100 (Hewlett-Packard, Slough, Berks., U.K.) as the support medium. The machine was set to give an increase of 0.5°C/min between 140 and 200°C. A flame (H₂/air)-ionization detector was used, and the elution of sample components was recorded by a Phillips PM 8000 chart recorder. The recorded results were traced on to paper and the various peaks were cut out and quantified by weighing; mannitol (20 nmol) was added to the samples as an internal standard, and, in addition, appropriate standard sugars (20 nmol of each) were examined.

T.l.c.

Samples (320 μ g of protein) of lavage material obtained from preparative column isoelectric focusing were examined on thin layers (0.25 mm) of silica gel on 20 cm $\times$ 20 cm glass plates (Macherey-Nagel, Düren, Germany) activated at 110°C for 30 min before use. The plates were developed with chloroform/methanol/water (100:42:6, by vol.) or twice, with thorough drying between developments, in chloroform/methanol/2.5 M-NH₃ (65:45:9, by vol.) as described by Esselman *et al.* (1972) for the separation of glycolipids and gangliosides. Cerebro-sides (type I), gangliosides (type III) and brain extract, all from bovine brain, were purchased from Sigma and used as marker substances. Glycolipids were detected with Molisch's reaction (Esselman *et al.* 1972) and gangliosides by means of resorcinol reagent (Svennerholm, 1957).

Results and Discussion*Enzymic and chemical analysis of macrophage membrane preparations*

Macrophage plasma membranes prepared by hypo-osmotic borate lysis (McColleston, 1970; Warley & Cook, 1973) and glass-bead chromatography were examined by electron microscopy. Material passing straight through the column of glass beads (fraction I) was found to consist of sheets of smooth membrane; contamination with mitochondria and cytoplasmic fragments, though greatly decreased as compared with the material obtained before column fractionation, was still present. Subsequent elution of the column with water yielded (in fractions II and III) sheets of membrane devoid of

any appreciable contamination by cytoplasmic fragments (see Plate 1).

The enzyme activities associated with the various fractions obtained from the macrophage are given in Table 1. In the case of fractions II and III the specific activity of 5'-nucleotidase, a presumptive plasma-membrane marker, was increased 3–8-fold and 3–12-fold respectively. The total percentage recovery of this activity in both fractions was 6.1 ± 4.7 (3). Although the recovery of this activity is lower than that found by Werb & Cohn (1972) for the plasma membrane of mouse peritoneal macrophages, the mean relative specific activity of 5.5 and 7.1 found in fractions II and III compares favourably with their values of 3.1–5.5. In one preparation, phosphodiesterase was examined as an alternative plasma-membrane marker. The percentage recovery of this activity in fractions II and III was 1.4 and 4.9 respectively, the relative specific activity being increased in fraction III only.

It is noteworthy that fractions II and III showed variable recoveries of NADH oxidoreductase (1.1–15.2%) between different batches of cells. Ferber *et al.* (1972) have suggested that this activity may be used as a reliable marker for endoplasmic reticulum, especially if glucose 6-phosphatase activity is low. These same authors (Ferber *et al.*, 1972) have, however, when working with fragments of plasma membrane from pig lymphocyte, found that the specific activity of this enzyme in the latter material is twice that present in the fraction designated as endoplasmic reticulum. The possibility that this enzyme is also a constituent of the macrophage plasma membrane cannot be overlooked.

The glass-bead-column chromatography was

Table 1. *Enzyme activities in membrane preparations and cell lysates*

Cell lysates and membrane fractions were prepared as described in detail in the text. The enzymes were assayed at 37°C except in the case of NADH oxidoreductase, which was determined at 20°C. Specific activities are quoted as nmol/h per mg of protein for all enzymes except NADH oxidoreductase and succinate dehydrogenase, which are given as μ mol/h per mg of protein. Numbers in parentheses represent the numbers of separate batches of cells fractionated and on which the various enzyme activities were determined. Abbreviation used: ND, no detectable activity.

Enzyme	Cell lysate specific activity	Post-column membrane fractions					
		I		II		III	
		Specific activity	Activity ratio*	Specific activity	Activity ratio*	Specific activity	Activity ratio*
5'-Nucleotidase	6.6 ± 3.6 (3)	28.2 (1)	2.7	30.4 ± 3.6 (3)	5.5	36.8 ± 2.4 (3)	7.1
Phosphodiesterase	11.6 (1)	7.5 (1)	0.6	6.9 (1)	0.6	66.3 (1)	5.7
NADH oxidoreductase	0.6 ± 0.4 (3)	1.6 (1)	7.2	1.3 ± 0.4 (3)	2.1	0.6 ± 0.1 (3)	1.0
Succinate dehydrogenase	2.0 (1)	2.7 (1)	1.4	2.0 (1)	1.0	2.1 (1)	1.0
Glucose 6-phosphatase	2.3 (1)	ND		ND		ND	
Acid phosphatase	44.0 ± 8.1 (3)	114.9 (1)	2.8	114.4 ± 3.6 (3)	2.6	29.2 (2)	0.7

* For each determination the ratio of the specific activity of the fraction to that of the starting lysate was measured. The values quoted in the Table are the mean values obtained from these determinations.

EXPLANATION OF PLATE I

Electron micrograph of membranes isolated from rabbit alveolar macrophages

A typical field of a thin section of the plasma-membrane fraction II prepared by using the isolation procedure described in the text is shown. The fraction consists predominantly of sheets and large vesicles of smooth membranes. Magnification $\times 32000$.

tested with one batch of cells for its effectiveness in removing the mitochondrial marker, succinate dehydrogenase. It was found that 74% of the available succinate dehydrogenase was recovered in the crude membrane fraction and that 82% of this activity was removed by passage through glass beads. Neither fraction II or III showed any purification of this marker, both having a specific activity similar to that of the cell lysate. Acid phosphatase is an important constituent of the phagolysosomes of the macrophage, and Werb & Cohn (1972) demonstrated that 35–84% of this activity is associated with this organelle in the mouse peritoneal macrophage. These workers (Werb & Cohn, 1972) also found 8–12% of the available acid phosphatase in the phagolysosome membrane and 8–12% in the plasma membrane. In the present experiments the percentage of acid phosphatase activity recovered in the crude membrane fraction was 18.1 ± 4.6 (3); glass-bead chromatography removed a significant quantity of this activity, with the percentage recovered in fractions II and III being 1.6 ± 0.6 (3).

In view of the above results, fractions II and III were regarded as being enriched with plasma membranes. As the quantities of macrophages, when compared with such cells as the human erythrocytes, are limited, these two membrane fractions were combined for chemical analyses. The yield of membrane expressed as a percentage of the total available protein was 1.4 ± 0.3 (3). Sialic acid content (expressed as *N*-acetylneuraminic acid) of this material was 17 nmol/mg of protein. Analytical polyacrylamide-gel electrophoresis of the combined fractions solubilized in sodium dodecyl sulphate indicated that five polypeptides were glycosylated and the apparent mol.wts. of these components were estimated as respectively 80000, 72000, 54000, 40000 and 28000.

Solubilization of macrophage membrane glycoproteins

In the preceding paper (Pratt & Cook, 1979) it was demonstrated that asialoglycoprotein of the human erythrocyte is not solubilized by Triton X-100, but that the reversible binding of borate to the carbohydrate groups enhances solubilization (Pratt & Cook, 1979). This method has the potential for solubilizing glycoproteins from membranes of other cell types. It would be a gross oversimplification to view all membrane glycoproteins as being necessarily in a similar class to the major sialoglycoprotein of the human erythrocyte. Thus less heavily sialylated membrane glycoproteins that might otherwise be refractory to solubilization by non-ionic detergent might be readily extracted from membranes after interaction with borate.

In view of the fact that the membrane fraction isolated from the rabbit alveolar macrophage is sialylated to a lesser extent than that of the human

erythrocyte, the use of Triton X-100 with borate was explored, a study of the constituents of the plasma membrane of the macrophage being of particular interest because of the involvement of this cell in endocytosis. Gross chemical analysis (Lowry *et al.*, 1951) of a batch of isolated macrophage plasma membranes, one half of which was treated with Triton X-100 in the presence of 50mM-Tris buffer, pH8.6, and the other with detergent in the presence of 50mM-borate buffer, pH8.6, revealed that 60 and 62% respectively of the available protein was solubilized. The relatively small quantities of solubilized material available from isolated macrophage plasma membranes were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and in addition by immunoelectrophoresis (see also below). Of the five well-discernible glycoproteins species shown to be present in the macrophage membrane fraction by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, only the glycoprotein of apparent mol.wt. 80000 was found to be solubilized with Triton X-100, adjusted to pH8.6. When the non-ionic detergent was used in the presence of 50mM-borate, pH8.6, the other four glycoproteins were also shown to be solubilized.

Effect of an inhibitor of phagocytosis on the endocytosis of latex particles by rabbit alveolar macrophages

The ability of a non-diffusible perchlorate-soluble fraction of rabbit lung wash-out fluid to inhibit the uptake of cholesterol particles and polystyrene-latex particles by alveolar macrophages has been extensively investigated by Ulrich & Zilversmit (1970). In the present work, which was aimed at studying the relationship of the components of the lung wash-out fluid to the macrophage plasma membrane, experiments were performed to check that the lung lavages under investigation possessed the biological activity described by Ulrich & Zilversmit (1970). The capacity of freeze-dried alveolar lavage components to inhibit the uptake of latex particles is detailed in Table 2. Consistent inhibition of particle uptake at 37°C was observed, and was found to be approximately proportional to the concentration of inhibitor present. In agreement with the results of Ulrich & Zilversmit (1970), the specific activity of the perchlorate-soluble inhibitor was slightly greater than that of the freeze-dried lung lavage.

Ulrich & Zilversmit (1970) demonstrated by means of kinetic data as well as by the use of enzyme digestion and metabolic inhibitors that uptake of latex particles at 0°C is due mainly to binding, whereas at 37°C uptake is due partly to binding and partly to ingestion. With our first batch of macrophages the uptakes of latex at 0 and 37°C were strikingly similar. Ulrich & Zilversmit (1970) also reported experiments in which they found an identical

Table 2. Effects of rabbit lung-lavage components on the uptake of latex particles by alveolar macrophage *in vitro*
 Separate batches of rabbit alveolar macrophages were used for each experiment and were obtained as described in the Materials and Methods section. The uptake of latex particles (0.822 μm diameter) over 2 h was measured in silicone-treated glass vials at 0 and 37°C; the complete incubation mixture contained in 2 ml of Krebs-Ringer phosphate buffer, pH 7.4, 2 mg of latex beads, 0.001% Tween 80, 8×10^7 cells and inhibitor. Two batches of cells were tested against dialysed and freeze-dried lung lavages and another two against perchlorate-soluble inhibitor, prepared as detailed in the text. Uptake of latex, as percentage of total added, was determined in duplicate for each experiment and the mean values are quoted in the Table.

Percentage inhibition =

$$\frac{\text{percentage uptake of latex in the absence of inhibitor} - \text{percentage uptake of latex in complete system}}{\text{percentage uptake of latex in the absence of inhibitor}} \times 100$$

Expt. no.	Incubation mixture	[Inhibitor] ($\mu\text{g/ml}$)	Uptake (%)		Inhibition (%)	
			37°C	0°C	37°C	0°C
	Dialysed lung lavage					
1	Complete mixture minus inhibitor	240	67.5	68.5	11.1	-2.9
	Complete mixture		60.0	70.5		
2	Complete mixture minus inhibitor	480	48.5	27.4	26.0	96.3
	Complete mixture		35.9	1.0		
	Perchlorate-soluble inhibitor					
3	Complete mixture minus inhibitor	360	37.0	10.9	21.4	93.6
	Complete mixture		29.1	0.7		
4	Complete mixture minus inhibitor	720	52.0	0.8	46.9	—
	Complete mixture		27.6	0.8

Fig. 1. Analytical gel isoelectric focusing of rabbit alveolar lavage material

Details of the sample preparation and staining are given in the Materials and Methods section. (a) and (b) Dialysed and freeze-dried lung lavage (0.8 mg of material used for each track); (c) and (d) inhibitor of phagocytosis prepared by treating lung lavage with perchlorate as described in the text (1.0 mg of inhibitor was used for each track). Sample blocks were applied midway between the anode and the cathode; the anode is at the top and the gel contains 1% (v/v) Triton X-100. A mixture of ampholytes designed to produce a pH 3.5–10 gradient was used, and the pH profile generated in this experiment is shown on the right of the gels. The appropriate regions of the gel were cut out for the different stains; tracks (a) and (c) were stained with Coomassie Blue and tracks (b) and (d) with periodate/Schiff reagent.

phenomenon. These authors (Ulrich & Zilversmit, 1970) suggest that, in those cases where the uptake of latex is found to be greater at 0 than at 37°C, this is probably due to a greater release of inhibitor by the macrophages at 37 than at 0°C.

Chemical composition of alveolar lavages

On examination by analytical-gel-electrophoresis techniques the lung lavage was found to contain a number of polypeptide species of apparent mol.wts. 102000, 74000, 68000, 60000 and 20000. Two of the polypeptide species of apparent mol.wts. (on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) 68000 and 60000 are glycosylated, as is indicated by staining with periodic acid/Schiff reagent (Zaccharius *et al.*, 1969). In analytical gel isoelectric focusing the glycosylated polypeptides are found to have pI values of 3.5–5.0, indicating there is some degree of micro-heterogeneity within these molecules (see Fig. 1). A number of non-glycosylated proteins are also found with pI greater than 5.0. The latter polypeptides are removed by precipitation with HClO₄, as judged by an examination by analytical isoelectric focusing of the inhibitor prepared by the method of Ulrich & Zilversmit (1970) (see Fig. 1).

The glycoproteins present in the lung lavage were isolated by preparative isoelectric focusing in the presence of 1% Triton X-100 at between pH 4.3 and 4.7 in the gradient, 9.3% of the applied protein being recovered in this region. An analytical gel isoelectric-focusing (see Fig. 2a) examination of the various fractions obtained from this preparative procedure demonstrated that in the presence of the detergent there is a clear separation between the glycosylated species and the polypeptides of pI greater than 5.0. In the absence of the detergent it appears that isoelectric focusing will not break up pH-independent component–component interactions, and aggregated material is found to be spread throughout the pH gradient. On examination by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the glycoprotein fraction obtained by isoelectric focusing was found to consist of the polypeptide species of apparent mol.wts. 68000 and 60000.

An analysis of the lung lavage and the various fractions isolated from this material is given in Table 3, together with the values quoted by Ulrich & Zilversmit (1970) for their inhibitor preparation. This perchlorate-soluble inhibitor has a very high content of glucosamine, and the values given for

Fig. 2. Analysis of fractions obtained from the preparative isoelectric focusing of rabbit alveolar lung lavage

A sample of rabbit alveolar lung lavage was fractionated by preparative isoelectric focusing over the pH range 2.5–6.0 in the presence of 1% (v/v) Triton X-100 as described in the Materials and Methods section. The various fractions obtained from the preparative isoelectric-focusing gradient were examined by (a) analytical-gel isoelectric focusing and (b) by diffused 'rocket' immunoelectrophoresis against guinea-pig anti-lavage serum. In the analytical isoelectric focusing (a), two stained gels are displayed, the gel slab on the left having been stained with periodate/Schiff reagent and the one on the right with Coomassie Blue. An effective pH gradient of 3.5–10.0 was generated in the separate gels. The lavage glycoproteins focused between pH 4.3–4.7 in 58 ml of the gradient, and the majority of the non-glycosylated proteins were recovered in 63 ml by the cathode solution; these fractions were dialysed extensively against distilled water and concentrated 3.9- and 4.2-fold respectively against Carbowax [poly(ethylene glycol)] before analysis. Sample blocks incorporating 200 µl of the concentrated fractions obtained from the preparative isoelectric focusing (pH values: a, 4.0–4.3; b, 4.3–4.7; c, 4.7–5.3; d, 5.3–5.5; e, 5.5–6.0; f, >6.0) were placed midway between the anode and the cathode; the anode is at the top. (b) Concentrated fractions obtained from the preparative isoelectric focusing were examined by diffused immunoelectrophoresis. Guinea-pig anti-lavage serum (30 µl) was incorporated into the agarose (12 ml) slab, and 20 µl samples of the concentrated fractions were examined. The notation a–f is the same as in (a); the six remaining wells to the right of well a are additional fractions focusing between pH 4.3 and the anodic acid. The well to the left of well f marked 'X' contains a sample of macrophage plasma membranes solubilized in 1% (v/v) Triton X-100/50 mM-borate, pH 8.6. There is a reaction of identity between components in the lung lavage and the macrophage membranes; the largest quantity of reacting material is found in the glycoprotein fraction b.

Table 3. Carbohydrate analysis of fractions obtained from rabbit lung lavages
The lung lavages were fractionated as detailed in the Materials and Methods section. Sialic acid was determined as *N*-acetylneuraminic acid by using the colorimetric procedure described in the text. Hexosamines were assayed by means of the amino acid analyser and the other saccharides as trimethylsilyl derivatives by means of g.l.c. In this latter method, mannitol (20nmol) was used as an internal standard with the samples under examination as well as with reference standards.

Sample	Saccharide ...	Content (nmol/mg of protein)							Reference
		Sialic acid	Fucose	Mannose	Galactose	Glucose	Glucosamine	Galactosamine	
Perchloric acid-soluble inhibitor of phagocytosis		80	9	66	173	42	925	Trace	Ulrich & Zilversmit (1970)
Dialysed lung lavage		47	17	56	51	27	77	Trace	Present work
Perchloric acid-soluble inhibitor of phagocytosis		33	52	59	101	127	45	—	Present work
Glycoprotein fraction from preparative isoelectric focusing		26	39	59	47	224	26	—	Present work

sialic acid and galactose are also higher than those found by us with comparable material. The glycoprotein fraction isolated by preparative isoelectric focusing has sialic acid, fucose and mannose contents similar to those of the inhibitor of phagocytosis prepared in the present work; however, the galactose content is decreased, whereas the amount of glucose present is almost double. The large amount of glucose is particularly noteworthy, since this saccharide is not usually found in significant quantities in membrane glycoproteins. Bole *et al.* (1975), however, have reported that a saline-soluble glycoprotein may be isolated from polyvinyl-sponge granuloma of guinea pigs that contains appreciable quantities of glucose. It is noteworthy that this glycoprotein has no effect on the phagocytic properties of guinea-pig polymorphonuclear leucocytes, but inhibits phagocytosis by guinea-pig macrophages. The above authors (Bole *et al.*, 1975) took care to avoid spurious contamination of their inhibitor preparation by chromatographic materials that contain polymers of glucose. It seems unlikely that the glucose found here is an artifact of the preparative isoelectric focusing, since glucose was found by us and by Ulrich & Zilversmit (1970) in the original lung lavage, and the focused samples were extensively dialysed before analysis. In addition, a sample of lung lavage material was subjected to isoelectric focusing on a gradient of ethanediol, and the glycoprotein fraction was found by the glucose oxidase method to contain 255nmol of glucose/mg of protein, a result in good agreement with the value for the fraction focused on a sucrose gradient. The presence of glycolipids such as glucosylceramide could account for the glucose found in the partially purified inhibitor. When the glycoprotein samples were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, no periodic acid/Schiff-reactive material was found in the region of the tracker dye, indicating that it is unlikely that any significant quantities of glycolipid are present. By using t.l.c. on silica gel we were unable to detect any contaminating glycolipids, including gangliosides, in the glycoprotein fraction obtained by preparative isoelectric focusing. Lung lavages are, however, likely to contain appreciable quantities of lung surfactant; this latter material is rich in phospholipid and is precipitated by trichloroacetic acid (Brown, 1962). A sample of dialysed lung lavage used in the present work was found to contain 95nmol of phosphorus per mg of protein, whereas the perchlorate-soluble inhibitor of phagocytosis and glycoprotein fraction prepared by isoelectric focusing were found to contain no detectable phosphorus. From this result it can be concluded that the analyses of the glycoproteins examined here are unlikely to represent contamination with lung surfactant.

Preparation of guinea-pig anti-lavage serum

Isoelectric focusing and immunoelectrophoresis may be combined in a modified two-dimensional Laurell (Cook, 1976) technique, and this provides a useful means of examining which components of the lung lavage cross-react with the guinea-pig antisera produced against this material. Results obtained with the cell-free rabbit alveolar lavage and guinea-pig antisera prepared in the present work have been used to illustrate the methodology involved with this modified Laurell technique. The guinea-pig antiserum was found to react with the acidic glycoproteins, forming two precipitin peaks that fuse, indicating that there is a reaction of identity between the components focusing in this region of the gel. This antiserum was used as a convenient tool for analysing by diffused 'rocket' immunoelectrophoresis the fractions obtained in the preparative isoelectric focusing of the rabbit lavage material (see Fig. 2b). The major amount of cross-reacting material is present in the glycoprotein fraction, as might be expected from the results with the modified two-dimensional Laurell technique.

It is noteworthy that the guinea-pig antisera prepared by us cross-reacts with the sera from both BCG-stimulated and unstimulated rabbits. We deduced by using the diffused 'rocket' technique that there is no immunological relationship between the glycoproteins isolated by isoelectric focusing with sera from unstimulated animals. The 'rockets' formed with sera from BCG-stimulated animals were difficult to interpret, since the precipitin lines disappeared in the region between the wells; however, no definite fused line was visible. Ulrich & Zilversmit (1970) reported the presence of an inhibitor of phagocytosis in the perchlorate-soluble fraction of rabbit serum, though clearly from the present results it is not possible to conclude that the inhibitors in lung lavage and serum are identical.

Relationships of macrophage lavage glycoprotein to membrane glycoproteins

Ulrich & Zilversmit (1970) postulated on the basis of composition data that their partially purified inhibitor resembles the carbohydrate-containing materials of the cell surface. A knowledge of the relationship of this soluble inhibitor to the plasma membrane is important when attempting to understand the mechanism of its action, and the availability of a guinea-pig antiserum directed towards the glycoproteins of the lung lavage provided an opportunity to study this point.

Plasma membranes isolated from a batch of alveolar macrophages (1.3 ml packed cell volume) were divided into two equal portions; one portion was extracted with Triton X-100 containing 50 mM-borate, pH 8.6, and the other portion was extracted

sequentially with the Triton X-100 adjusted to pH 7.4, and then the 100000g pellet was re-extracted with detergent adjusted to pH 8.6. In each case 200 μ l of extraction solution was used and portions (up to 7 μ l) were examined against the guinea-pig antisera in 'rocket' immunoelectrophoresis. Only in the case of the material extracted with detergent in the presence of borate was a precipitin line detected, the distance migrated by the tip of the 'rocket' being linearly related to the amount of material applied to the sample well.

It is noteworthy that a precipitin reaction was only obtained under conditions demonstrated by analytical gel electrophoresis to result in the solubilization of all of the glycoproteins present in the membrane fraction. The relationship of the glycoproteins isolated from the lung lavage by preparative isoelectric focusing to the membrane glycoproteins solubilized with Triton X-100/borate was examined by mono and tandem two-dimensional immunoelectrophoresis. Samples of the isolated lavage glycoproteins were subjected to electrophoresis ahead of the membrane extract in the first dimension and then the separated components allowed to migrate by electrophoresis into guinea-pig anti-lavage serum in the second dimension; only a single precipitin line, showing a complete reaction of identity between the membrane and lavage material, was obtained. In addition, fused 'rocket' immunoelectrophoresis of the above materials into the guinea-pig antiserum also resulted in a reaction of identity (see Fig. 2b). Also, a reaction of identity was obtained in this technique between the detergent/borate-solubilized membrane, fractionated lung lavage glycoproteins and material present in the supernatant fluid of lung wash-outs that had been subjected to centrifugation at 100000g for 1 h. This latter result indicates that the guinea-pig anti-lavage serum is unlikely to be reacting solely with membrane fragments that might be present in the lung wash-outs.

Conclusions

It has been suggested that the inhibition of endocytosis by soluble glycoproteins of the lung lavage is due to the covering of the exogenous particle with material resembling that present on the macrophage cell surface (Ulrich & Zilversmit, 1970). Such particles might then show no greater affinity for binding to the macrophage than the phagocytes exhibit for each other. The present demonstration of a relationship between the glycoproteins of the macrophage membrane and the lung lavage indicates that such an explanation is entirely feasible. An alternative mechanism of action might be found in the recent work of Stahl *et al.* (1978), which shows that alveolar macrophages have a cell-surface receptor that binds glycoproteins with terminal sugars with the mannose or glucose configuration,

while excluding those with terminal galactose residues. The glycoproteins examined in the present work contain appreciable quantities of glucose, and it is possible that the binding of considerable quantities of such glycosubstances to the cell periphery might serve to sterically hinder 'recognition sites' for the exogenous test particles. The availability of the mild method described here for fractionating the lung lavage makes possible the isolation of sufficient material to test this latter possibility experimentally.

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