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Cytophilic Antibody

I. NATURE OF THE MACROPHAGE RECEPTOR

M. JEAN DAVEY AND G. L. ASHERSON

Department of Bacteriology, London Hospital Medical College, London

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Summary. Cytophilic antibody was produced in guinea-pigs by single injections of sheep erythrocytes in Freund's complete adjuvant. The nature of the receptor for this antibody was studied by treating macrophages with various reagents and determining changes in their ability to take up cytophilic antibody, as shown by the subsequent adherence of sheep red cells.

Receptor activity was destroyed by phospholipase A, iodine and periodate: it was reduced by lanthanum and uranyl ions, but not by calcium ions, although all these ions are able to interact with lipids. It was also reduced by poly-L-lysine.

The receptor was not affected by pH in the range 5·4–9·0 or by the presence of EDTA, heparin, chloroquine or hydrocortisone. Neuraminidase and lipase had little effect but proteolytic enzymes appeared to increase the uptake of cytophilic antibody.

It was concluded that a phospholipid or phospholipoprotein is an important part of the macrophage receptor for cytophilic antibody.

INTRODUCTION

The term cytophilic antibody was introduced by Boyden and Sorkin (1960, 1961) to describe a circulating antibody in rabbits that adhered to spleen cells from various animals and was detected by the adsorption of radioactive antigen onto the antibody-coated cells. Boyden (1964) described a cytophilic antibody produced in guinea-pigs by immunization with sheep red cells in Freund's complete adjuvant. Sheep red cells adhered to macrophages coated with this antibody.

Sorkin (1963, 1964) studied the nature of the receptor on spleen cells for cytophilic antibody using rabbit antisera produced by repeated intravenous injections of human serum albumin. He found that the activity of the receptor was destroyed by proteolytic enzymes, and reduced by ether, chloroform, lipase and freeze-drying. Sorkin suggested that the receptor might be a lipoprotein.

The present paper describes similar experiments on the nature of the receptor of peritoneal exudate macrophages in guinea-pigs using cytophilic antibody produced by single injections of sheep red cells in Freund's complete adjuvant. The finding that the receptor was resistant to proteolytic enzymes but inactivated by phospholipase A suggested that it might be a phospholipid or phospholipoprotein.

MATERALS AND METHODS

Guinea-pigs

Guinea-pigs weighing 200–600 g were used.

Media

Hanks's tissue-culture balanced salt solution buffered with 0·007 per cent bicarbonate, M/200 Gomori's Tris or M/150 Sörensen's phosphate (final concentrations) was used to collect and dilute macrophage suspensions and to prepare enzyme solutions.

Dulbecco phosphate buffered saline with added calcium–magnesium solution (PBS: Oxoid) was used for washing both guinea-pig macrophages and sheep red cells and for dissolving many of the reagents. Dulbecco saline without calcium and magnesium (PBS.A) was used for experiments with a chelating agent.

To avoid precipitate or complex formation heavy metal solutions were diluted in unbuffered 0·8 per cent sodium chloride.

Sheep red blood cells

Sheep red cells (RBC) preserved in Alsever's solution (Burroughs Wellcome) were washed three times with PBS before use. It was necessary to use RBC not more than 1 week after collection as older cells adhered to glass and made reading of cytophilic antibody tests extremely difficult.

Sera

Sheep RBC in Freund's complete adjuvant were prepared by emulsifying equal volumes of 30 per cent RBC and a mixture of 4 parts of paraffin oil with 1 part of Arlacel A: killed human tubercle bacilli were added to a final concentration of 1 mg/ml. Into the four footpads of each guinea-pig was injected 0·05 ml of the emulsion. After 3 weeks the animals were bled to obtain sera.

Two standard sera were used throughout these experiments at dilutions of 1/20 to 1/80 that is, at concentrations at least three times higher than their cytophilic antibody titres which were 1/128 and 1/320 respectively. No attempt was made to determine what fraction of the total antibody content was cytophilic antibody or to make a study of the physico-chemical nature of the antibody adhering to macrophages in the two standard sera and the term cytophilic antibody was operationally defined as antibody which under the condition of testing adhered to macrophages.

Enzymes

Proteolytic enzymes were dissolved in 0·001 N HCl and diluted in Tris-buffered Hanks's solution of appropriate pH immediately before use. To activate papain and ficin 0·02 M cystein hydrochloride and 0·01 M EDTA were used. Commercial samples of crystalline α-chymotrypsin and TPCK-treated trypsin (Seravac Laboratories, South Africa), papain (British Drug Houses Ltd), ficin (Koch-Light Laboratories) and pronase (California Biochemical Research Co.) were used. Unpurified neuraminidase was used in the form of *Vibrio cholerae* culture filtrates (Wellcome Research Laboratories).

Wheat-germ lipase, and *Naja naja* cobra venom as a source of phospholipase A, were obtained (Koch-Light Laboratories). The venom was reconstituted in M/150 phosphate buffer pH 6·0, boiled for 15 minutes to destroy the activity of contaminating proteolytic enzymes and diluted for use in buffered Hanks's solution pH 7·5. Commercial samples of phospholipases C and D were obtained (Sigma Chemical Co.) and fresh *Bacillus cereus* culture filtrates were used as a further source of phospholipase C (Slein and Logan, 1965).

Enzyme treatments of macrophages were all carried out at 36°, except in the case of phospholipase D which was used at 26°.

Other reagents

The effect of pH was determined by using Hanks's solution buffered to pH values in the range 5·4–9·0 for all stages of the cytophilic antibody test after adherence of the macrophages to the glass surface. This method was also used to test the effect of ethylene diamine tetra-acetate (EDTA) dissolved in Dulbecco PBS.A.

Poly-L-lysine was dissolved and diluted in PBS. Uranyl acetate, calcium chloride and lanthanum chloride were dissolved in distilled water and diluted in 0·8 per cent sodium chloride to maintain approximately isotonic solutions. These reagents were used to treat macrophages at room temperature for 1 hour. Heparin and lysolecithin solutions were prepared in PBS and used to treat both macrophages and sera for 1 hour at room temperature.

Periodic and peracetic acids were used in M/5 acetate buffer pH 5·6. Iodine was dissolved in 2 per cent aqueous potassium iodide and diluted in 0·8 per cent sodium chloride. Methanol was diluted with distilled water. Formaldehyde solutions were prepared using PBS. These reagents were used to treat macrophages for 10 minutes at room temperature.

Chloroquine sulphate and hydrocortisone sodium succinate were dissolved in phosphate buffered Hanks's solution pH 7·2, containing 10 per cent normal guinea-pig serum and 100 units/ml crystalline benzylpenicillin. Macrophages were treated for 6 hours at 36°.

Macrophage preparations

Peritoneal exudate cells were harvested from normal guinea-pigs 4 days after the intraperitoneal injection of 10 ml of light liquid paraffin or 10 per cent sodium caseinate. The cells were washed twice in the cold before use.

Lipid extract was prepared by suspending the cells in freshly-boiled distilled water and adding this suspension dropwise to 9 volumes of a mixture of 2 parts of methanol and 1 part of chloroform. Nitrogen was bubbled through the mixture, which was left overnight. The solution was filtered and the solvent removed in a rotary evaporator at 37°. The residue was taken up in a small volume of toluene which was evaporated and the residue dissolved in methanol. This was added quickly to 9 volumes of saline to give a milky suspension. On some occasions the toluene step was omitted.

Polysaccharide extract. The method of Westphal, Lüderitz and Bister (1952) was used. The aqueous layer of the hot phenol (65°) was dialysed and freeze-dried. No alcohol precipitation was undertaken.

Subcellular fractions. Macrophages were washed once in 0·25 M sucrose containing 0·002 M EDTA and disrupted in a Potter–Elvehjem homogenizer. The nuclei were deposited by centrifugation at 500 *g* for 12 minutes and the supernatant was centrifuged at 11,000 *g* for 12 minutes. The supernatant from this was centrifuged at 95,000 *g* for 60 minutes. The deposits (nuclei, mitochondria, fluffy layer and microsomes) were suspended in sucrose and stored at −20° (Cohn and Wiener, 1963).

These fractions were tested for their ability to absorb cytophilic antibody. Each preparation was mixed with the usual dilution of serum and incubated at room temperature for 1 hour. Insoluble absorbents were removed by centrifugation at 50,000 *g* for 10 minutes and the supernatants were tested for residual cytophilic antibody.

Cytophilic antibody test

Macrophages were obtained by washing out the peritoneal cavity of a bled normal guinea-pig with 20 ml of Hanks's solution containing 0·007 per cent bicarbonate and 2·5

u/ml heparin. This suspension was diluted 1/3 to 1/5 in phosphate buffered Hanks's solution pH 7·2.

Simple chambers were prepared as described by Boyden (1964). In each cup was placed 0·3 ml of macrophage suspension and the slides were incubated in a moist chamber at 36° for 1–2 hours during which time the cells settled out and adhered to the glass surface. The macrophages were washed by removing the fluid with a fine-tipped pipette and refilling the cups, usually with PBS. This was replaced by 0·1 ml of a test reagent and the slides were incubated appropriately. The cells were then washed three times, generally with PBS. In tests on EDTA and heavy metals all washings were done using PBS. A or unbuffered saline.

Next, 0·1 ml of a standard serum dilution was placed in each cup and the slides were incubated at room temperature for 1 hour. The cells were then washed five times with PBS and 0·1 ml of a 1 per cent suspension of sheep RBC was added to each cup. After standing again for 1 hour at room temperature the macrophages were washed once and the cups sealed by filling them with PBS, placing a coverslip on the top and blotting carefully. The slides were then inverted and left for at least 10 minutes before examining under the microscope.

In every experiment three chambers were treated with each reagent: two of these then received positive cytophilic antibody serum, and the third a similar dilution of normal guinea-pig serum. Control slides were always set up using untreated macrophages and macrophages treated with appropriate solvents.

Counts were made of the number of red cells attached to 100 randomly distributed macrophages in each preparation. In positive controls there were usually 800–1200 RBC/100 macrophages: in negative controls there were only 30–100 RBC/100 macrophages.

Counts obtained from treated macrophages were expressed as percentages of those from suitable concurrent controls. All observations described were repeated three or more times, but on some occasions results were only recorded qualitatively and the numerical data were based on less than three counts.

RESULTS

A standard system was used to investigate the effect of enzymes and other reagents on the receptor for cytophilic antibody. Peritoneal macrophages were allowed to adhere to glass coverslips in simple chambers and were treated either with a test reagent or an appropriate control solution. The macrophages were then treated with a standard serum containing cytophilic antibody, and finally with a suspension of sheep red cells. The effect of treatment on the uptake of cytophilic antibody was assessed by the percentage ratio of red cells adhering to treated and untreated macrophages.

PROTEOLYTIC ENZYMES

Proteolytic enzymes such as trypsin, chymotrypsin, pronase and ficin consistently failed to reduce the activity of the receptor. Indeed, all these enzymes increased the number of red cells adhering. This was probably due to increased uptake of cytophilic antibody rather than increased stickiness of the macrophages. Thus, few red cells adhered to macrophages treated with proteolytic enzymes when they were subsequently exposed to a normal serum

or to a serum containing haemagglutinating but no cytophilic antibody, produced by immunization with red cells in Freund's incomplete adjuvant. These results are presented in Table 1.

TABLE 1

THE EFFECT OF ENZYMES ON THE MACROPHAGE RECEPTOR FOR CYTOPHILIC ANTIBODY

Macrophage treatment			Percentage activity* of the receptor	
			Time of macrophage treatment	
Enzyme	Concentration (mg/ml)	pH	1 hour	3 hours
Trypsin	0·2	8·0	148	138
α-Chymotrypsin	0·2	8·0	132	116
Papain	1·0	7·2	152	91
Ficin	1·0	7·2	126	116
Pronase	1·0	7·5	n.d.	188
Lipase	1·0	8·0	n.d.	96
Neuraminidase	Solution as supplied	5·6	n.d.	116
Phospholipase A	0·1	7·5	11	n.d.
Phospholipase D	1·0	5·4	83	75

* Results are expressed as $\frac{\text{RBC count on treated macrophages}}{\text{RBC count on untreated macrophages}} \times 100$, thus low figures indicate inactivation of the receptor. n.d., Not done.

PHOSPHOLIPASES

Phospholipases A, C and D catalyse the hydrolysis of lecithins and certain other phosphoglycerides. Phospholipase A yields a lysolecithin and an unsaturated fatty acid, phospholipase C a diglyceride and phosphoryl choline and phospholipase D a phosphatidic acid and choline.

Naja naja venom destroyed the activity of the macrophage receptor. Table 2 shows that the active principle behaved like phospholipase A. It resisted 100° for 15 minutes at pH 6

TABLE 2

THE EFFECT OF VARIOUS TREATMENTS ON THE ABILITY OF *Naja naja* VENOM TO INACTIVATE THE MACROPHAGE RECEPTOR

Treatment of *Naja naja* venom	Percentage activity* of the receptor	
	Time of macrophage treatment	
	30 minutes	60 minutes
None	2	2
100° for 15 minutes at pH 6	10	2
100° for 15 minutes at pH 8	n.d.	90
0·5 mM EDTA†	124	64
1 mM sodium fluoride†	5	6
1 mM zinc sulphate†	95	99
1 mM lead acetate†	61	18

High figures indicate inactivation of the venom. n.d., Not done.

* See footnote to Table 1.

† Venom previously heated in phosphate buffer at pH 6 for 15 minutes at 100° to destroy proteolytic enzymes.

but not at pH 8 and was inhibited by 0·5 mM EDTA though not by 1·0 mM sodium fluoride. Inhibition of the enzyme by certain heavy metals was consistent with the findings of Magee, Gallai-Hatchard, Sanders and Thompson (1962) for pancreatic phospholipase A.

The effect of phospholipase C from bacterial culture filtrates could not be determined as the solutions removed macrophages from the glass surface. The one preparation of phospholipase D tested did not affect the receptor.

Lipase, neuraminidase and neuraminidase even when preceded by pronase, were also ineffective.

OTHER REAGENTS

The effect of several agents was tried to provide collateral evidence for the phospholipid nature of the receptor. The activity of the receptor was reduced by poly-L-lysine, uranyl ions and lanthanum, although calcium ions, which are also able to react with phospholipids (Bangham, Pethica and Seaman, 1958), had no effect. Oxidizing agents such as

TABLE 3

THE EFFECT OF OTHER REAGENTS ON THE MACROPHAGE RECEPTOR FOR CYTOPHILIC ANTIBODY

Macrophage treatment		Percentage activity* of the receptor		
		Dilution of reagent		
Reagent	Initial concentration	1	1/10	1/100
Poly-L-lysine	1 mg/ml	n.d.	30	44
Uranyl acetate	N/10	17	35	70
Calcium chloride	N/1	99	n.d.	n.d.
Lanthanum chloride	N/1	41	96	119
Periodic acid	1 per cent w/v	4	72	99
Peracetic acid	10 per cent v/v	1	95	92
Iodine	0·1 per cent w/v	0	2	42
Chloroquine sulphate	20 μg/ml	85	n.d.	n.d.
Hydrocortisone sodium succinate	20 μg/ml	102	n.d.	n.d.

Low figures indicate inactivation of the receptor. n.d., Not done.

* See footnote to Table 1.

TABLE 4

THE EFFECT OF OTHER REAGENTS ON THE MACROPHAGE RECEPTOR FOR CYTOPHILIC ANTIBODY

Macrophage treatment		Result of cytophilic* antibody test
Reagent	Concentration	
pH 5·4–9·0	—	+
EDTA	0·5 mM	+
Lysolecithin	0·1 mg/ml	−
Formaldehyde	0·4 per cent w/v	+
Methanol	10–50 per cent	+
Methanol	100 per cent	−
Heparin	1 mg/ml	+

* A negative result indicates inactivation of the receptor.

iodine, buffered periodic acid and peracetic acid destroyed receptor activity. The receptor was usually resistant to 0·4 per cent w/v formaldehyde and 50 per cent methanol but not to 100 per cent methanol. Lysolecithin had a variable effect but at a concentration of 0·1 mg/ml usually destroyed receptor activity. Heparin had no effect whether added to the macrophages or to the serum at a concentration of 160 u/ml. Receptor activity was not appreciably reduced by treatment with hydrocortisone and chloroquine. There was no effect of pH in the range 5·4–9·0 or of 0·5 mM EDTA. Similar results were obtained by Berken and Benacerraf (1966). These results are summarized in Tables 3 and 4.

MACROPHAGE EXTRACTS

Westphal polysaccharide extract at a concentration of 10 mg/ml did not absorb cytophilic antibody. An initial positive result with a chloroform–methanol extract of macrophages could not be confirmed.

There was some absorption of cytophilic antibody by crude nuclear, mitochondrial and microsomal fractions. These fractions did not destroy the macrophage receptors when incubated with macrophages. In particular the mitochondrial fraction (which should have contained lysosomes) had no effect.

DISCUSSION

The experiments show that the receptor for cytophilic antibody is inactivated by phospholipase A but not by proteolytic enzymes. This suggests that the receptor is a phospholipid, or a phospholipoprotein in which the protein moiety is protected. The other results are compatible with this interpretation. Thus among their other actions, iodine, periodate and peracetate react with double bonds and may alter the unsaturated fatty acids of lecithin. Uranyl and lanthanum ions and presumably poly-L-lysine associate with the phosphate group of lecithin and might be expected to alter its biological action through charge reversal (Bangham *et al.*, 1958). The lack of effect of calcium ions is unexplained.

The failure of phospholipase D to destroy the receptor may be due to steric effects or inappropriate surface charge of the substrate, as electrostatic conditions are known to affect the activity of some phospholipases (Dawson, 1964). Long has found that this enzyme will not attack the lecithin of intact red cells (personal communication).

Cell fractionation studies showed that the receptor occurred in crude nuclear, mitochondrial and microsomal fractions, but not in the soluble cellular protein. This suggests that the receptor is attached to the cell membrane and perhaps other membranes.

The results provide further evidence that the cytophilic antibody produced in rabbits immunized with human serum albumin is different from the cytophilic antibody produced in guinea-pigs by immunization with sheep red cells in Freund's complete adjuvant. The rabbit antibody, which is measured by the binding of radioactive labelled human serum albumin, adheres to polymorphs and lymphocytes (Sorkin, 1964) and there are at least two distinct types (Blazkovec and Sorkin, 1966). The receptor for this type of cytophilic antibody is sensitive to proteolytic enzymes. In contrast, the guinea-pig cytophilic antibody, measured by the adherence of sheep red cells, will only attach to macrophages (Jonas, Gurner, Nelson and Coombs, 1965) and the receptor is insensitive to proteolytic enzymes. The question of heterogeneity of different types of cytophilic antibody is important in the

consideration of its biological role (Blazkovec, Sorkin and Turk, 1965; Berken and Benacerraf, 1966) and the attachment of cytophilic antibody to the surface of a macrophage suggests that it may be readily pinocytosed.

There are a few other examples in biology of lipid receptors and of biological agents acting on lipid components of membranes. Fischer (1964) considered that lysolecithin was produced when antibody and complement acted on target cells, while Soule, Marinetti and Morgan (1959) showed that the haemolytic action of mumps virus is associated with alteration of the red cell lipids. Acylated polysaccharides attach to red cells and the requirement for lipid combined with the polysaccharide suggests that the red cell receptor for lipopolysaccharides may be a lipid (Tsumita and Ohashi, 1964). Finally some of the receptors for 5-hydroxytryptamine are lipid (see Marchbanks, Rosenblatt and O'Brien, 1965).

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