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Effects of Phagocytosis and Colchicine on the Distribution of Lectin-Binding Sites on Cell Surfaces

(polymorphonuclear leukocytes/concanavalin A/*Ricinus communis* agglutinin)

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ABSTRACT **The effect of phagocytosis on lectin binding to plasma membranes of polymorphonuclear leukocytes was examined. The specific activities of binding sites of concanavalin A and *Ricinus communis* agglutinin (defined as μg of lectin bound per 100 μg of membrane protein) were measured on isolated membranes; they decreased in parallel with phagocytosis. Our data suggest that this removal occurs by concentration of binding sites into internalized membrane. Colchicine and vinblastine, which did not inhibit phagocytosis, prevented the selective removal of lectin-binding sites from the surface. It was also shown that at 37° lectins induce their own internalization. This property was used to define operationally three classes of lectin receptors, one of which is most extensively removed from plasma membrane during phagocytosis. Based on other morphological studies in which it is shown that before phagocytosis the surface distribution of concanavalin-binding sites is random, it is inferred that phagocytosis alters surface topography by inducing the selective movement of binding sites into membrane undergoing internalization and that colchicine-sensitive proteins are essential for this imposed topographical reorganization.**

According to the fluid mosaic model of cell surfaces, proteins are free to diffuse in the membrane of cells and thus should assume a random or homogeneous distribution (1). Yet, several studies have indicated that certain components (2) and functions (3) dependent on proteins are disposed over the surface nonrandomly. The resolution of this paradox is suggested by some morphological studies of the distribution of concanavalin A (Con A)-binding sites (CABS) on transformed cells (4, 5); the inherent distribution of CABS *is random* but CABS may be induced to cluster (i.e., assume a nonrandom distribution) by addition of exogenous Con A. We have also presented evidence that colchicine-sensitive proteins may modify the movement of CABS (6, 7) and membrane-transport proteins (8).

During phagocytosis, particles are enveloped by cytoplasmic membrane which is then withdrawn into membrane-bound intracellular vesicles. Suitable particles can stimulate the internalization of a large portion of the membrane (3). Thus, phagocytosis causes the removal of an operationally defined region of the cell surface, permitting examination of the properties and composition of the residual surface membrane. Removal of membrane from the surface during phagocytosis of plastic beads does not lead to a decrease in the transport of nonelectrolytes, i.e., transport carriers are not present on the portion of cell membrane that is internalized (8). After colchicine treatment, however, phagocytosis leads to a marked reduction in transport, which presumably represents the internalization of transport proteins (8).

In this study we have investigated whether phagocytosis leads to removal or to preservation of the receptors for the lectins, Con A and *Ricinus communis* agglutinin (RCA), on cell surfaces.

METHODS

Enzyme Assays. β-Glucuronidase (β-D-glucuronide glucuronohydrolase; EC 3.2.1.31) activity is measured from the hydrolysis of phenolphthalein glucuronide (9) and acid and alkaline phosphatase activities from the hydrolysis of *p*-nitrophenylphosphate during incubation at pH 5.2 and 9.0, respectively. 5′-Nucleotidase (5′-ribonucleotide phosphohydrolase; EC 3.1.3.5) is measured from the hydrolysis of [^{3}H] AMP during incubation with enzyme at pH 9 in the presence of β-glycerophosphate. Unreacted AMP is removed by coprecipitation with $Ba(OH)_2$ and $(Al)_2(SO_4)_3$ (10), and released [^{3}H]adenosine is measured in the supernatant solution. Protein is measured by the method of Lowry *et al.* (11).

Cells. Rabbit polymorphonuclear leukocytes (PMN) are obtained from a sterile peritoneal exudate (12) and are suspended in modified Hanks' medium at 4°.

Phagocytosis. The PMN phagocytized a finely divided emulsion of paraffin oil droplets coated with bovine-serum albumin prepared as described (13). Oil red O is dissolved in the paraffin oil for quantification of phagocytosis (13). Cells are incubated in 20 volumes of modified Hanks' medium containing 1 mM glucose, 0.5 mM $CaCl_2$, 2 mM $MgSO_4$, and 1 volume of emulsion. They are subsequently collected by centrifugation and washed to remove excess emulsion. Fig. 1 (*top*) shows that ingestion is rapid over the first 10 min of incubation at 37°, reaching a maximum after 20 min when each cell contains between 3 and 12 intracellular oil-filled vesicles.

Subcellular Fractionation. For isolation of membranes and phagocytic vesicles, cells (150 to 250 × 10^6) are homogenized in 3–4 ml of 0.34 M sucrose in 7-ml Dounce homogenizers (Kontes). A discontinuous gradient consisting of 1 M sucrose (3 ml) overlaid with 0.40 M sucrose (5 ml) is prepared in 13-ml tubes. The homogenate is layered above the 0.40 M sucrose, and the tube is filled with 0.25 M sucrose. The tubes are cen-

Abbreviations: Con A, concanavalin A; CABS, concanavalin A binding sites; RCA, *Ricinus communis* agglutinin; LBS, lectin-binding sites; PMN, polymorphonuclear leukocytes; CBP, colchicine-binding proteins.

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trifuged for 35 min at 110,000 × g in the SW 41 head of the Beckman model L3-50 ultracentrifuge. During this time the oil-filled phagocytic vesicles collect above the gradient and the plasma membranes are retained at the 0.40–1.0 M sucrose interface. The plasma membrane fraction is characterized by its high activity of 5′-nucleotidase (14, 15). The specific activities of 5′-nucleotidase in the various fractions of a typical run expressed in pmoles hydrolyzed in 30 min/100 μg of protein were: vesicles, 1.11; membranes, 1.61; supernatant, 0.0009; and pellet (including some intact cells), 0.11. Recovery of the activity from the total homogenate was essentially complete, and about 70% of the activity could be accounted for as the sum of the vesicle and membrane fractions. No β-glucuronidase activity was detectable in the membrane fraction, indicating the absence of lysosomal contamination. The phagocytic vesicles show 5′-nucleotidase activity, as expected for a fraction derived from plasma membrane. They also show appreciable β-glucuronidase and acid and alkaline phosphatase activities. These are characteristic lysosomal enzymes (16) and indicate that the phagocytic vesicles have undergone extensive lysosomal fusion.

Lectins. RCA was the generous gift of Dr. G. Nicolson. Con A (3× crystalline) was purchased from Miles Yeda. The lectins were iodinated by the method of Helmkamp *et al.* (17).

RESULTS

Binding of Lectins Is Specific. The binding to isolated membranes of both Con A and RCA at all concentrations used was inhibited 96% by addition of their specific hapten sugar inhibitors (50 mM α-methyl-D-mannose, Con A; D-galactose, RCA).

Lectin Sites Are Labeled to Saturation under Our Experimental Conditions. A 30-min labeling period at 4° was used in all experiments unless otherwise noted. Lectins are bound but not internalized at this temperature, while an extensive internalization of bound lectin occurs at 37°. At concentrations of 5 μg/ml of Con A and above, longer incubations did not increase binding. Although higher Con A concentrations gave more rapid adsorption, the maximal levels attained did not exceed those with 5 μg/ml. RCA adsorption appeared to be more rapid, and maximal values were attained after only 10 min of incubation, although a 30-min period was routinely used. Identical conditions gave maximal labeling of cells or isolated membranes after phagocytosis, and virtually no lectin was released into the medium during membrane isolation.

Effect of Phagocytosis on Lectin Binding. Lutton (18) found that after phagocytosis there was a large decrease in the amount of Con A taken up by monolayers of whole cells on coverslips. We have confirmed this result using incubation of cell suspensions with lectin. In order to quantify this binding of lectins to cell surfaces, and to differentiate the process of binding from internalization of lectins, we used an approach based on the determination of the specific activity of lectin bound to isolated membranes. The principle is: if lectin receptors are distributed homogeneously over the surface, then internalization of membrane by phagocytosis should not alter the specific activity of the lectin receptors isolated with the remaining membrane. If lectin distribution is not random, selective inclusion or exclusion of receptors should lead to a fall or rise, respectively, in the lectin specific activity of the noninternalized membrane.

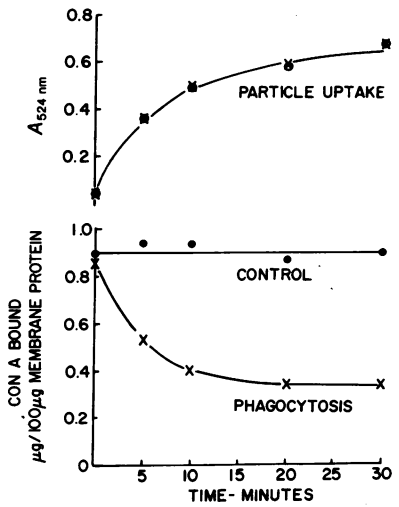

FIG. 1. *Top:* A plot of the extent of phagocytosis as a function of time with (O——O) or without (×——×) colchicine. Cells were treated with the alkaloid at 5 μM for 30 min before initiation of phagocytosis. *Bottom:* Effect of phagocytosis on specific activity of CABS of isolated membranes. 4 × 10^6 cells/ml of medium were incubated without (*control*) and with (*phagocytosis*) emulsion for the times indicated. Cells were then centrifuged, washed, and resuspended in modified Hanks' medium containing 7 μg/ml of labeled Con A and incubated at 4° for 30 min. Membranes were then isolated, and the specific activity of Con A bound was determined.

Fig. 1 (*bottom*) shows the effect of phagocytosis on the specific activity of CABS expressed as μg of Con A bound per 100 μg of membrane protein on isolated membranes. Phagocytosis is interrupted at the times indicated, and CABS is measured in the isolated membrane fractions. There is a sharp decrease in the specific activity of CABS with a time-course that parallels the time-course of phagocytosis (Fig. 1, *top*). The fall in specific activity ranges between 40 and 60% (15 experiments), depending on the number of particles ingested by the cells, which varies somewhat from day to day. No decrease in binding occurs in cells incubated at 37° in the absence of emulsion, in the presence of bovine-serum albumin alone, or in cells incubated with emulsion in phagocytic medium where the 2 mM $MgSO_4$ is replaced by 5 mM EDTA to prevent phagocytosis. When emulsion is removed after 5 min of phagocytosis and cells are further incubated in the absence of particles, no further decrease in CABS occurs. Hence, the continued presence of emulsion is required to produce the maximal decline in CABS, i.e., phagocytosis does not set into motion some general process leading to the removal of CABS from the cell surface.

In these experiments the membrane is isolated after incubation of whole cells with lectin at 4°. However, the specific activity of membrane-bound lectin is very similar whether lectin is incubated with whole cells and the membrane then isolated (0.85–1.10 μg/100 μg of membrane protein in 15 experiments) or directly with isolated membranes (1.04 μg/100 μg of membrane protein; average of three experiments). This observation supports published evidence that these lectin receptors are present only on the outer surface of the plasma membrane (19).

The decrease in lectin binding is not due to cleavage of surface sites by enzymes released during phagocytosis, since incubation of cells in medium obtained from cell suspensions that had undergone phagocytosis and from which remaining

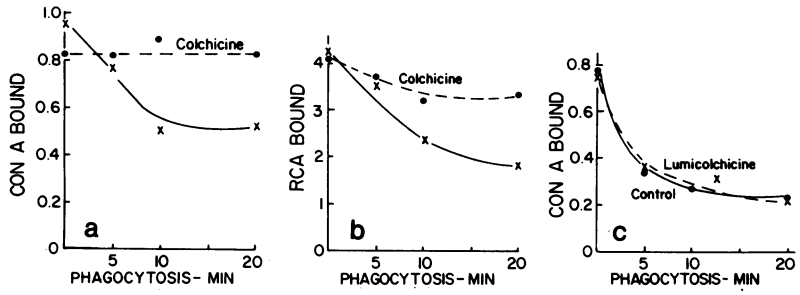

FIG. 2. Effect of colchicine alkaloids on changes in membrane LBS produced by phagocytosis. Cells were preincubated with 5 μM alkaloid (*dashed line*) or modified Hanks' medium alone (*solid line*) for 30 min before addition of emulsion and during phagocytosis. (*a*) Effect of colchicine on CABS. Labeled with 7.5 μg/ml of Con A. (*b*) Effect of colchicine on RCA-binding sites. Labeled with 20 μg/ml of RCA. (*c*) Effect of lumicolchicine on CABS. Labeled with 6.3 μg/ml of Con A.

emulsion is removed by centrifugation or from which Mg^{2+} is removed by addition of EDTA results in no decrease in CABS specific activity. It is also unlikely to reflect a shedding of CABS during phagocytosis since the loss of bound Con A into the medium from cells labeled at 4° and then incubated at 37° in the presence or absence of emulsion is very small and almost identical with and without phagocytosis. A similar reduction in RCA binding to cell surfaces occurs during phagocytosis. By contrast, the specific activity of 5′-nucleotidase of isolated membranes is unchanged with phagocytosis, indicating its homogeneous surface distribution.

Two arguments can be marshalled against the hypothesis that the specific activity of lectin-binding sites (LBS) in plasma membrane is reduced due to the introduction into the surface of new membrane that does not contain LBS, e.g., by the fusion of lysosomes with the membrane during phagocytosis. (*a*) Less total lectin is absorbed to whole cells after phagocytosis. (*b*) The specific activity of 5′-nucleotidase is unchanged with phagocytosis, although lysosomes have no 5′-nucleotidase (15). This could result from a parallel exclusion of 5′-nucleotidase from internalization with insertion of new membrane protein (which would dilute LBS). However, lysosomes have a high density of both Con A- and RCA-binding sites. Under comparable labeling conditions, the membranes of isolated lysosomes bind Con A and RCA to a density of about 10-fold that of plasma membranes.

We conclude that the introduction of new membrane during phagocytosis is not quantitatively important with respect to the mass of surface protein internalized. Lectin receptors are thus selectively removed from the surface during phagocytosis, very probably by inclusion in membrane-bound intracellular vesicles.

Effect of Colchicine on the Internalization of Lectin Receptors Induced by Phagocytosis. Colchicine and vinblastine are plant alkaloids that bind at low concentrations to protein subunits of microtubules (20, 21) and perhaps to other proteins that have similar pharmacologic specificities. These will be referred to as colchicine-binding proteins (CBP). When PMN are incubated for 30 min with colchicine (5 μM) before addition of emulsion, the amount of phagocytic ingestion of oil droplets is unchanged (Fig. 1, *top*). However, unlike untreated cells, there is relatively little decrease in the specific activity of the LBS that remain on the cell surface (Fig. 2*a*). Cells pretreated with 5 μM vinblastine also show a much higher residual binding of Con A to postphagocytic membranes. The results (Fig. 2*b*) indicate that colchicine treatment also inhibits the removal of RCA receptors from the plasma membrane during phagocytic internalization of oil droplets. Lumicolchicine, a derivative of colchicine which does not bind to microtubular proteins but does bind to nucleoside transport proteins (22, 23), has no effect on the removal of lectin receptors (Fig. 2*c*).

Fig. 2*a* also shows a decrease of about 10–20% (15 experiments) in the amount of Con A that binds to cells exposed to alkaloids alone (without emulsion). (Note intersection with ordinate.) However, colchicine does not affect the ability of control cells to bind RCA (Fig. 2*b*). This specific effect of colchicine on CABS appears to reflect a direct action of colchicine on the membrane, since isolated membranes exposed to 5 μM colchicine for 30 min show decreased CABS compared to control membranes.

Heterogeneity of Lectin Receptors. Since lectins are specific only for certain carbohydrate endgroups, it must be supposed that lectin receptors are chemically heterogeneous. In the following experiments this heterogeneity of receptors is defined operationally into three classes: (*a*) those specific for Con A only, (*b*) those specific for RCA only, and (*c*) those in which the chemical determinants for both lectins are apparently associated with the same molecular species. These experiments are based on the observation that when Con A is preincubated with cells at 37°, only 36% of adsorbed lectin can be eluted by α-methyl-D-mannoside, whereas 90% is eluted at 4°. Similarly, when cells are incubated with RCA at 37°, very little lectin is released by specific hapten sugars, while with preincubation at 4° essentially all lectin can be eluted. The internalized lectin is recovered in the lysosomal fraction of control cells and in the phagolysosomal fraction of postphagocytic cells when they are labeled at 37°. These fractions are unlabeled after incubation of whole cells with lectin at 4°. Thus, at 37° lectin is removed from the surface perhaps by pinocytosis, but remains accessible to the eluting sugar at 4°. The residual population of CABS on the membrane (20–40% in six experiments) after prolonged incubation at 37° or 4° may represent receptors that cannot be internalized after binding with lectin.

Whole cells were first incubated at 37° with nonradioactive lectins, washed, and labeled at 4° (i.e., labeled under conditions in which negligible internalization occurred). Membranes were then isolated, and the specific activity of adsorbed lectin was determined. The results in Table 1 show that cells preincubated in modified Hanks' medium alone and then labeled with [^{125}I]Con A (7 μg/ml) at 4° bind 0.89 μg of Con A per 100 μg of membrane protein. If the cells are preincubated at 37° with a high concentration (50 μg/ml) of nonradioactive

TABLE 1. *Effect of preincubation of whole cells with lectins on subsequent labeling at 4°*

Line	Incubations at 37° a	Incubations at 37° b	Incubations at 4°	μg of [125I]-lectin/100 μg of membrane protein	No. of expts.
				[125I]Con A	
(1)	MH	None	—	0.89	15
(2)	Con A	None	—	0.03	4
(3)	Con A	αMM	—	0.17	2
(4)	RCA	None	—	0.45	4
(5)	RCA	gal	—	0.40	2
(6)	—	—	RCA	1.00	2
				[125I]RCA	
(7)	MH	None	—	6.99	6
(8)	RCA	None	—	0.39	4
(9)	RCA	gal	—	0.41	2
(10)	Con A	None	—	4.13	4
(11)	Con A	αMM	—	4.55	2
(12)	—	—	Con A	6.20	2

250 × 10^6-cell aliquots were incubated for two 30-min periods at 37° in 25 ml of modified Hanks' solution (*MH*) in the presence or absence of lectins (50 μg/ml of Con A; 30 μg/ml of RCA). They were washed and incubated further in 40 ml of modified Hanks' medium with or without competing sugars [50 mM α-methyl-D-mannoside (*αMM*); 50 mM D-galactose]. Cells were collected, washed, suspended in 10 ml of modified Hanks' medium and incubated 30 min at 4° with [125I]lectin (7.5 μg/ml of Con A; 10 μg/ml of RCA). About 400–500 μg of membranes were subsequently collected and counted. The results of Tables 1 and 2 are given for a single experiment. Repeated experiments gave the same qualitative relationships but differed in absolute value.

lectin, this binding is reduced by 96% (compare lines 1 and 2). Incubation of cells with α-methyl-D-mannoside to remove surface-bound lectin before labeling restores a small proportion of the original lectin-binding capacity (line 3). This is in contrast to the effects of similar treatment of cells labeled at 4°, which completely restores the binding capacity. Therefore, incubation with lectin appears to have promoted a very extensive removal of CABS from the cell surface.

Similarly, the binding of [125I]RCA to cells is reduced by 95% as a result of preincubation at 37° with 30 μg/ml of RCA (compare lines 7 and 8), and treatment of the cells with galactose does not restore the ability of the cells to bind the radioactive lectin (line 9). Hence, RCA also promotes the internalization of its own receptors at 37°.

When cells are pretreated with *RCA* at *37°* and then labeled with [125I]*Con A* at 4° there is an approximate 50% decrease in CABS (compare lines 4 and 1). This is not reversed by treatment of cells with galactose before labeling, indicating that the inhibition of Con A binding is a result of the internalization of its binding sites by RCA. Similarly, cells pretreated with Con A bind 40% less RCA than control cells (compare lines 10 and 7). These data indicate that there is at least one population of receptors that has binding sites for both lectins and is removed from the cell surface after binding with either lectin, probably by internalization. This population could correspond either to separate but closely linked macromolecules or, since RCA and Con A bind to different sugars, to single macromolecules containing binding sites for both lectins.

TABLE 2. *Effect of colchicine and phagocytosis on lectin binding*

Line	Incubations at 37° a	Incubations at 37° b	Incubations at 37° c	μg of [125I]-lectin/100 μg of membrane protein	No. of expts.
				[125I]Con A	
(1)	None	None	—	0.89	15
(2)	Emulsion	None	—	0.34	15
(3)	Emulsion	Con A	—	0.04	4
(4)	Emulsion	RCA	—	0.36	2
(5)	Colchicine	None	—	0.80	15
(6)	None	RCA	—	0.45	2
(7)	Colchicine	RCA	—	0.45	2
(8)	Colchicine	Emulsion	—	0.45	15
(9)	Colchicine	Emulsion	RCA	0.32	2
				[125I]RCA	
(10)	None	None	—	6.99	6
(11)	Emulsion	None	—	2.22	6
(12)	Emulsion	RCA	—	0.43	2
(13)	Emulsion	Con A	—	2.61	4
(14)	Colchicine	None	—	6.85	6
(15)	None	Con A	—	4.13	4
(16)	Colchicine	Con A	—	3.62	4
(17)	Colchicine	Emulsion	—	4.06	6
(18)	Colchicine	Emulsion	Con A	2.90	4

As Table 1, except cells were preincubated with or without colchicine and with or without emulsion in 100 ml of phagocytosis medium during the first two 30-min incubations.

Further evidence that the reduction of labeling of one lectin after preincubation with the other at 37° is due to internalization of a common receptor rather than simple mechanical interference is obtained from experiments in which cells are preincubated at 4° when *no* internalization takes place. When cells are incubated at 4° with RCA and then treated with [125I]Con A (or Con A then [125I]RCA) there is no decrease in the specific activity of lectin bound to the plasma membrane (compare lines 1 and 6 or 7 and 12, Table 1).

Whereas preincubation with either lectin at 37° reduces the amount of binding of the same lectin by 95%, binding of the other is reduced maximally about 50%. We infer from this that, in addition to surface receptor molecules with linked Con A- and RCA-binding sites, there are two other classes specific for Con A or RCA alone.

Effect of Phagocytosis on Internalization of Different Populations of Lectin Receptors. With three classes LBS defined, we can now ask whether they are removed from the plasma membrane differentially during phagocytosis (Table 2). In cells that have extensively phagocytized there is a large reduction in the binding of both lectins as compared to the binding by control cells (compare lines 2 and 1, 11 and 10) as already shown in Figs. 1 and 2. This binding can be reduced to less than 10% of control by incubation of cells after phagocytosis at 37° with the same lectin as in control cells (compare lines 3 and 2, 12 and 11). However, one lectin no longer promotes the internalization of receptors for the second lectin (compare

lines 4 and 2, 13 and 11). This indicates that the joint binding sites for both RCA and Con A have been selectively removed, presumably by internalization, during phagocytosis and that the residual sites are specific for just one lectin. The effects of colchicine on operationally defined populations of lectin receptors that are altered by phagocytosis can now be examined. Colchicine treatment in control cells does not alter the reciprocal inhibition of Con A binding by RCA nor of RCA binding by Con A (lines 6 and 7, 15 and 16). The membranes from cells after phagocytosis, which were pretreated with colchicine before phagocytosis, show a higher capacity to bind both lectins than untreated cells Fig. 2; and compare lines 8 and 2, 17 and 11, Table 2). However, with colchicine the reciprocal effects of pretreatment of one lectin by binding by the second are retained after phagocytosis (compare lines 9 and 8, 18 and 17). These data indicate that some of the joint receptors, which are normally removed during phagocytosis, remain on the surface of colchicine-treated cells.

DISCUSSION

At 37° binding of the lectins Con A and RCA to the PMN surface leads to their removal from the surface, presumably by endocytosis. Likewise, Con A bound to lymphocytes under certain conditions is gathered into a "cap" which is internalized (24).

The present study shows that phagocytosis itself leads to selective removal of lectin receptors. Our evidence further indicates that the major receptors that are removed are a subclass bearing both Con A- and RCA-binding sites. This selective removal appears to be due to inclusion of receptors into internalized membrane, since it only occurs when the cell is actively phagocytizing and ceases abruptly when the particles are removed. We hoped to confirm this proposed internalization of surface receptors by labeling cells with lectin before exposure to the emulsion and following the incorporation of label into the phagolysosomes. However, the inhibition of phagocytosis by high concentrations of Con A (26) and the internalization of Con A–receptor complexes that occurs at 37° independently of phagocytosis precluded these experiments. Binding studies with isolated phagolysosomes were also precluded since the vesicle membrane is in reverse orientation to the plasma membrane so that receptors are inwardly directed and inaccessible to assay. In addition, new lectin receptors are probably introduced into the vesicles during lysosomal fusion.

This internalization could occur if the lectin receptors are normally gathered into areas that are internalized during phagocytosis, or alternatively if they normally have a random distribution but can be concentrated into these regions during phagocytosis. Our morphological evidence indicates clearly that the inherent distribution of the total population of CABS is random in several cell types examined, including PMN (ref. 4, and Ukena, Rosenblith, M. J. Karnovsky, and Berlin, unpublished observations). Moreover, after removal of the joint RCA and Con A receptors, residual CABS are also randomly distributed, indicating that the inherent distribution of all classes of CABS is random. Thus, just as exogenous Con A may induce clustering of receptors (4, 5) and its own internalization (this study), by analogy the phagocytized particle may serve to induce the concentration of lectin receptors into certain regions.

CBP appear to be essential for the selective removal of lectin receptors from the plasma membrane. This seems difficult to reconcile with the concept that CBP anchor surface elements geographically, a possibility originally suggested by us (6–8) and recently elaborated by Edelman (25). This paradox is resolved if we postulate that CBP, rather than anchoring surface elements, are required for their *directed movement.* It may be that colchicine disrupts microtubules or other CBP that are connecting links between lectin receptors and contractile elements. The specific movement of CABS from their random distribution into internalized membrane during phagocytosis may require this coupling with intracellular elements, while for cap formation lectin receptors need be released from these connections in order that they be aggregated by lectins that serve as exogenous crosslinking agents.

The use of lectin receptors, coupled with the phagocytic cystem, has thus given us a chemical means for characterization of surface topography under dynamic conditions in which large areas of membrane can be modified and then removed for analysis. The results show clearly a topographical heterogeneity of lectin receptors induced by exogenous particle contact. CBP appear to be essential for this induction.

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