THE EFFECT OF PHAGOCYTOSIS AND SPREADING ON MACROPHAGE SURFACE RECEPTORS FOR CONCANAVALIN A

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INTRODUCTION

Phagocytosis involves attachement of a particle to the cell surface (12), followed by engulfment of the particle within a membrane-bounded vacuole (7, 20). It can be assumed that the plasma membrane is reduced by the amount interiorized. Much morphological evidence supports this assumption; however, there is little direct evidence on the fate of membrane surface markers during phagocytosis.

Tsan and Berlin (19) provided intriguing evidence that several membrane transport carriers are not internalized during phagocytosis. They suggested that during membrane interiorization, transport and phagocytic sites go their separate ways, an idea consistent with Singer's fluid mosaic

model of the plasma membrane (15). Considering the relatively large size of the latex beads used, this mechanism would require a very rapid skimming of all transport sites during phaogcytosis.

In this study, binding of concanavalin A (Con A) to macrophages was examined before and after large portions of the surface membrane became internalized by phagocytosis. The effect of extensive macrophage spreading on the binding of the agglutinin was also studied.

MATERIALS AND METHODS

Animals

Mice of the CFW strain, of both sexes, weighing 20-25 g, were used for a source of peritoneal macrophages. To obtain stimulated macrophages, mice were injected intraperitoneally with 1 ml of thioglycollate medium (Difco Laboratories, Detroit, Mich)., and cells were collected 5 days later in phosphate-buffered saline (PBS).

Chemicals

Con A, crystallized three times, was obtained from Miles Laboratories, Inc. (Elkart, Ind.). Trypsin, cycloheximide, iodoacetic acid, and α-methylmannoside (MAM) were from Sigma Chemical Co. (St. Louis, Mo.), Cleland's reagent (DTT) was from Calbiochem (LaJolla, Calif.), and cytochalasin B was from Gallard-Schlesinger Chemical Mfg, Corp. (Carle Place, N. Y.); ^{125}I was from Nuclear-Chicago Corp. (Des Plaines, Ill.). Polyvinyl toulene latex (PVT), 2.02 μm diameter, was from Dow Chemical Co., (Midland, Mich.), and TiO_2 was from E. I. DuPont de Nemours and Co., Inc. (Wilmington, Del.).

Media

PBS was prepared as previously (13). Dulbecco's modified Eagle's medium, with or without bicarbonate and fetal calf serum (FBS), was obtained from Grand Island Biological Co. (Grand Island, N. Y.). Heart infusion broth was from Difco Laboratories.

Macrophages were cultivated in Dulbecco's medium alone or with the addition of 10% FBS or 10% vol/vol of 2.5% heart infusion. For phagocytosis, the medium was Dulbecco's without bicarbonate with 10 mM Tris at a final pH of 7.2.

Iodination of Con A

Con A was iodinated according to McFarlane (9), and was extensively dialyzed against PBS. The iodinated Con A had a specific activity of 10^7 cpm/mg,

and agglutinated sheep erythrocytes (0.1%) down to 0.1-0.5 μg/ml. The agglutination was reversed with 50 mM MAM (6).

Collection of Macrophages and Cultivation

Thioglycollate-stimulated or unstimulated peritoneal macrophages were aseptically collected and allowed to attach to albumin-coated 9 $\times$ 22 cover glasses (13). After attachment, the monolayers were rinsed in PBS and placed in 35 $\times$ 10 mm Falcon plastic tissue culture dishes (Falcon Plastics, Div of Bioquest, Oxnard, Calif.) with 1-2 ml of medium. In phagocytosis experiments, 1,000 μg/ml PVT (2.3 $\times$ 10^8 beads/ml) or 50 μg/ml TiO_2 were added to modified Dulbecco's media plus 10 mM Tris, and the cells were incubated with the particles for 1 h at 37°C. In one experiment, the number of PVT particles taken up was estimated at 38 $\pm$ 13 ($\bar{X} \pm$ SD n, = 20). In experiments where cells were maintained in culture after phagocytosis, the monolayers were removed from the PVT and control dishes, rinsed three times in PBS to remove excess PVT, placed in culture dishes with the desired media, and incubated in a CO_2 incubator for 1-48 h.

In experiments designed to study the effects of spreading on the amount of Con A binding, monolayers were incubated for 1 h at 37°C in Dulbecco-Tris with or without 200 μg/ml trypsin, 2 mM DDT, or 5 μg/ml cytochalasin B before the binding assay. Maximal spreading of cells usually occurred within 30 min of incubation.[1]

Con A Binding Assay

Binding assays were conducted at 18°C in a moist chamber. The chamber contained cover slip support racks, which held the monolayers to be assayed. The procedure for binding assay was as follows: Monolayers were removed from the culture dish, washed three times in PBS, and placed horizontally in the chamber. 50 μl of $[^{125}I]$Con A were added to each monolayer for 15 min. The monolayers were washed three times in PBS to remove excess Con A, and fixed in 2% glutaraldehyde. Radioactivity was measured in a Nuclear-Chicago Ultrascaler II gamma counter (Nuclear-Chicago Corp.). In all experiments, backgrounds were run on cover slips without cells in order to account for nonspecific binding of $[^{125}I]$Con A. This value was then deducted from experimental values to give a final corrected determination. Binding of the agglutinin was found to be linearly proportional to the number of cells applied to the cover slips, ranging between 1 $\times$ 10^4 and 1 $\times$ 10^5 cells per cover slip.

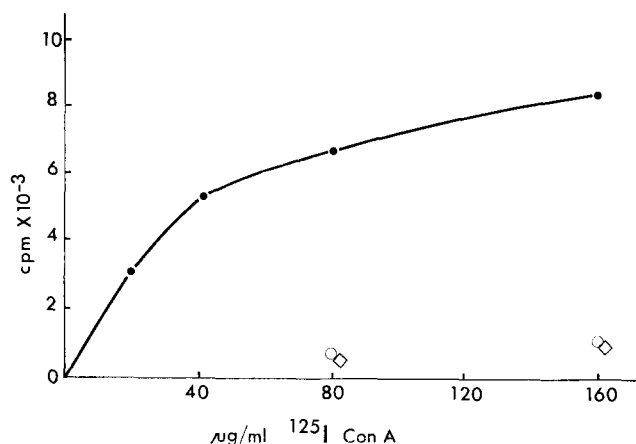[1] Rabinovich, M., and M. J. DeStefano. 1973. Macrophage spreading in vitro. I. *Exp. Cell Res.* In press.

FIGURE 1 Dose response of [^{125}I]Con A added to mouse peritoneal macrophage monolayers. O = 50 mM MAM added before [^{125}I]Con A. ◇ = cold Con A added for 15 min before [^{125}I]Con A. Each point represents an average of three experiments.

Validation of the Assay

In order to determine if endocytic uptake of Con A could have affected the results, macrophages were incubated with Con A alone or with 1 mM iodacetate or 2 mM sodium azide. No significant difference was found between macrophages incubated with Con A in the presence or absence of the inhibitors. In a typical experiment, while incubation with Con A alone resulted in 6,300 ± 330 ($\bar{X}$ ± SE, n = 10) cpm, macrophages treated with Con A and iodoacetate bound 5,970 ± 310 cpm; in another experiment, Con A binding was 5,290 ± 238 cpm for control, and 5,636 ± 158 for macrophages incubated with Con A and azide, respectively. Therefore, it can be assumed that membrane transport had no detectable effect on the binding of [^{125}I]Con A.

Other experiments indicate that most of the counts are associated with the macrophage surface. Macrophages were treated with 40 μg/ml Con A for 15 min at 18°C, rinsed in PBS, and incubated for 20 min in PBS with 100 mM of either MAM or D-galactose. Counts on both cover slips and medium showed that whereas 75% of the counts were eluted by MAM, only less than 10% were recovered by incubation with galactose.

Dose Response of [^{125}I]Con A Binding

Preliminary experiments were conducted to determine [^{125}I]Con A binding at different concentrations. Fig. 1 is a typical binding curve for [^{125}I]Con A bound to macrophages attached to cover slips. The data suggests that near maximal binding was obtained between 40 and 80 μg/ml [^{125}I]Con A. A similar response was obtained with stimulated macrophages.

In all subsequent assays, 40 μg/ml [^{125}I]Con A were used. Binding was inhibited 90% by 50 mM MAM or by pretreatment with equivalent amounts of unlabeled Con A.

Data were subjected to analysis of variance, and P was determined by the t test.

RESULTS

The Effect of Macrophage Spreading on [^{125}I]Con A Binding

Macrophages were induced to spread on glass by incubation with DTT or trypsin. In order to determine whether trypsin could influence the binding of Con A under conditions in which extensive spreading was not induced by the enzyme, macrophages were also incubated with trypsin together with cytochalasin B. This drug markedly inhibits induced macrophage spreading.[1] It can be seen in Fig. 2 that binding of Con A to macrophages treated with trypsin, DTT, cytochalasin B, or cytochalasin plus trypsin did not differ significantly from binding to control macrophages. In other experiments, no significant difference was found in the Con A binding in suspensions of macrophages treated with 2 mM DTT for 30 min.

The Effect of Phagocytosis on [^{125}I]Con A Binding

Initial experiments were conducted on monolayers of thioglycollate-stimulated macrophages that were allowed to phagocytize TiO₂ or PVT
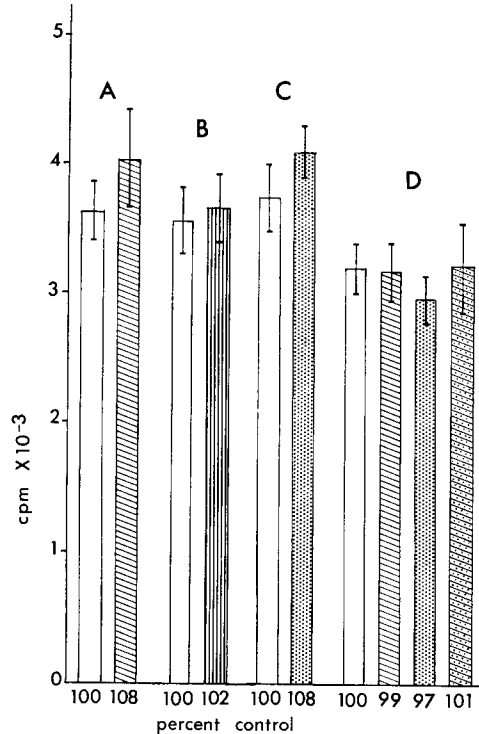
FIGURE 2 Effect of spreading on the binding of [125I]Con A to macrophages. Results are given in an average count per minute per cover glass ± SE and as a percent of controls. (A and B) Macrophages induced to spread with 200 μg/ml trypsin (diagonal lines) or 2 mM DTT (vertical lines), respectively. (C) Macrophages treated with 5 μg/ml cytochalasin B (dots). (D) Macrophages treated with trypsin, with cytochalasin B, or with both (diagonal lines + dots). Open bars: Macrophages incubated in saline Tris-Mg++ medium alone. In experiments A, B, and C, each group consisted of ten monolayers. In experiment D four replicates used per treatment.

for 1 h, removed from the incubation medium, washed in PBS, and assayed for [125I]Con A binding. Because PVT particles were also bound to macrophages, it was necessary to examine whether particle attachment alone could reduce the binding of Con A. Accordingly, in several experiments 1.0 mM iodoacetate was also added to the incubation medium to inhibit uptake. Cells were preincubated with the inhibitor 15 min before the addition of particles. Data in Table I demonstrate that macrophages, after extensive phagocytosis, bound 25–35% less Con A when compared to control cells. Furthermore, macrophages incubated with 10^{-3} M iodoacetate and PVT or TiO_2

showed little change in [125I]Con A binding as compared with controls. For example, binding of [125I]Con A to control cells was 14,769 cpm, cells after phagocytosis of PVT, 9,730 cpm (35% reduction), and cells in the presence of iodoacetate plus PVT, 14,215 cpm (similar to controls). Iodoacetate inhibited phagocytic uptake but not particle attachment as evidenced by the "rosette" appearance of PVT bound to the macrophages. These data suggest that membrane receptors for Con A are internalized during the ingestion step of particle engulfment.

The following experiments attempted to examine the recovery of Con A binding by incubating macrophages for different time periods after particle uptake and then assaying with [125I]Con A. In addition it was of interest to examine the need for serum and the effect of a protein synthesis inhibitor.

Binding of [125]Con A to cells after phagocytosis and incubation in Dulbecco's medium alone for up to 24 h is shown in Table II. Full binding did not recover within 24 h after phagocytosis.

Fig. 3 shows that the binding of Con A to macrophages that were allowed to phagocytize and were then postincubated for 8 h in medium containing serum, recovered initial levels of Con A binding. Partial recovery of Con A binding occurred by $4\frac{1}{2}$ h in serum and to a lesser extent in serum-free media. In addition, when cycloheximide was present during the postincubation, binding of Con A was similar to that immediately after phagocytosis. Furthermore, substitution of heart infusion media for serum produced a similar effect. As an alternative to serum, heart infusion was used because it does not contain macromolecular components precipitable by trichloroacetic acid.

These experiments show that serum or heart infusion broth accelerates the recovery of Con A binding and that the recovery is inhibited by cycloheximide.

DISCUSSION

The main finding of this investigation was that binding of Con A was unaffected by spreading of macrophages on glass, whereas it was significantly reduced after particle ingestion. Furthermore, the recovery of binding after phagocytosis required several hours in an enriched medium and was inhibited by cycloheximide.

Macrophage spreading has been compared to

TABLE I

Binding of 40 µg/ml $[^{125}I]Con$ A to Stimulated Macrophages after Phagocytosis of PVT or TiO_2 for 1 h in Dulbecco's Medium with or without 10^{-3} Iodoacetate (IAA)

	^{125}I cpm M ± SD				P	
Material phagocytosed	(a) Control*	(b) After phagocytosis	(c) Phagocytosis with 10^{-3} M IAA added	Binding (a vs. b)	(a vs. b)	(c vs. b)
Experiment I (PVT)	14,769 ± 1,200 (100) 8**	9,730 ± 1,174 (65) 8**	14,215 ± 1,576 (100) 6**	−35%	<0.001	<0.01
Experiment II (PVT)	15,426 ± 673 (100) 9**	11,994 ± 1,000 (75) 9**	16,806 ± 1,800 (108) 9**	−25%	<0.02	<0.001
Experiment I (TiO₂)	13,860 ± 2,700 (100) 8**	9,510 ± 573 (68) 3**	12,964 ± 1,017 (99) 8**	−32%	<0.05	
Experiment II (TiO₂)	7,636 ± 1,100 (100) 9**	5,292 ± 1,200 (69) 9**		−31%	<0.02	

Numbers in parentheses are percent of control.
* Controls include cells incubated without particles ± IAA.
** Number of replicates.

TABLE II

Binding of 40 µg/ml $[^{125}I]Con$ A to Macrophages that were Postincubated in Fresh Medium without Serum 1–24 h after Phagocytosis of PVT

H in vitro after phagocytosis*	^{125}I cpm M ± SD			
	0	1	8	24
(a) Controls (no phagocytosis)	23,856 ± 1,470 (100) 5**	21,396 ± 1,390 (100) 4**	24,742 ± 1,190 (100) 4**	22,724 ± 1,640 (100) 4**
(b) PVT (phagocytosis)	18,111 ± 1,020 (78) 5**	16,427 ± 1,440 (75) 4**	19,353 ± 1,960 (78) 4**	18,810 ± 1,290 (82) 4**
Binding (a vs. b)	−22%	−25%	−22%	−18%
P (a vs. b)	<0.01	<0.05	<0.05	<0.05

Numbers in parentheses are percent of control.
* Macrophages were incubated with PVT particles for 1 h at 37°C.
** Number of replicates.

the attempted phagocytosis of a particle of very large diameter (11). Electron microscope studies have shown that the macrophage surface is thrown into numerous folds or ridges (4). It has been suggested that these folds represent reserve membrane, as the surface of the macrophage is smoothened when the cell spreads (4). Our results are consistent with this hypothesis since they indicate that the numbers of membrane receptors for Con A are similar for macrophages in the round as in the spread configurations. Caution must be taken in interpreting this type of data since it was not possible to determine whether the Con A binding beneath the cell was similar to that above the cell. However, experiments on cells in suspension support these observations.

The data also indicate that trypsin does not remove Con A membrane receptors, or exposes more receptors for binding. This is of interest since trypsin has been reported both to unmask agglutinin receptors (3), and to remove other types of membrane receptors (8, 21).

a. The reduction of $[^{125}I]Con$ A binding after phagocytosis is best explained by the interiorization of outer membrane as phagocytic vacuoles. Therefore, binding sites for Con A did not behave like the transport sites studied by Tsan and Berlin (19). Particle attachment to the cell surface did
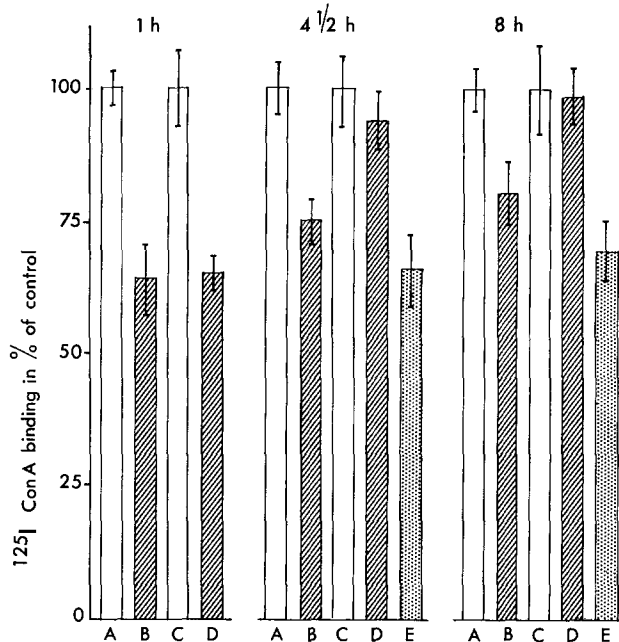
FIGURE 3   Binding of [^{125}I]Con A to macrophages allowed to phagocytize and postincubated in different media and for different time periods. Results are expressed in [^{125}I]Con A bound in percent of control monolayers incubated without PVT beads. Standard errors are indicated and each line gives the average of four replicate monolayers. Bars A and C represent controls incubated in Dulbecco's alone or with 10% FBS, respectively. Bars B and D indicate Con A bound by monolayers that phagocytized PVT and were postincubated in Dulbecco's or in Dulbecco's + FBS, respectively. Bar E represents binding after phagocytosis and postincubated in Dulbecco's + FBS + 7 μg/ml cycloheximide.

not by itself reduce Con A binding. This was evidenced by the fact that Con A binding was not reduced when particle engulfment was inhibited by iodoacetate. The reduction in binding of Con A immediately after termination of phagocytosis was around 25–30%. Because an estimate of the actual surface of the macrophage is not possible at this time, it is difficult to ascertain whether the reduction in Con A binding was indeed proportional to the fraction of the surface membrane interiorized.

Topological studies have demonstrated differences in the distribution of Con A receptors for certain cell types (3, 10), and Smith and Hollers (15) have shown that fluoresceinisothiocyanate (FITC)-labeled Con A-bound lymphocytes mainly in the posterior region. Preliminary experiments in this laboratory revealed that macrophages exposed to fluorescein-labeled Con A demonstrated a uniform pattern of fluorescence. It would be of interest to know the precise distribution of Con A binding sites on macrophages in relation to the postulated

phagocytic receptors, particularly because Con A has been shown to inhibit phagocytosis of PVT particles (1).

Our model appears to be consistent with the hypothesis that *de novo* membrane synthesis or repositioning of membrane components may take place after extensive phagocytosis. These events may be dependent upon serum factors or heart infusion broth added to the medium. This is not surprising since it is difficult for macrophages to synthesize certain proteins in serum-less media (5, 16, 17). Furthermore, our results are consistent with Werb and Cohn's observations on cholesterol exchange (21) and plasma membrane 5'-nucleotidase marker (22) suggesting that macrophages may interiorize much of their surface after phagocytosis, followed by a rest period during which synthesis of new membrane receptor is necessary before a new cycle can be initiated. Also of interest is the finding that phagolysosomes and plasma membranes share common antigens (18). It has been estimated that in *Acanthamoeba* the surface

membrane turnover after endocytosis is several-fold per hour, and that the membrane is probably recycled soon after the endocytotic event (2). If repositioning of membrane components was to take place, one must assume that a membrane pool must be available for recycling. In our experiments, the macrophages did not appear to degrade or exocytose the injested PVT. Therefore, under conditions where the PVT remains in the phagocytic vesicle, de novo synthesis appears to take precedence over repositioning of membrane components, a possibility that fits with the inhibitory effect of cycloheximide.

SUMMARY

Binding of [^{125}I]Con A by mouse peritoneal macrophages was determined before and after extensive spreading or particle uptake. Spreading induced by DDT or trypsin did not influence the binding of the agglutinin, whereas after phagocytosis binding was significantly reduced.

Macrophages cultivated for 8 h after phagocytosis recovered Con A binding provided serum or heart infusion broth was present. The recovery was prevented by the protein synthesis inhibitor cycloheximide.

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