Macrophage Pinocytosis: The Removal and Resynthesis of a Cell Surface Factor[1] (35843)

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The uptake of particulates and soluble proteins by endocytotically active cells proceeds in two steps (1–3): binding to the cell surface, the attachment phase, and invagination of the surface membrane bearing the bound material, the ingestion phase. In the case of the ameba, the initial interaction is known to require ionic binding of positively charged macromolecules to acidic groups at the cell surface (3). Macrophages, however, do not exhibit a requirement for a positive charge on the ingested proteins (4), so that simple ionic forces cannot explain binding of proteins to this cell type.

Immunoglobulin and complement receptors provide a means of attaching antigenic proteins and particulates to the cell surface, but macrophages are quite capable of taking up exogenous proteins in the absence of any deliberately induced antibody, and *in vitro* in the absence of any serum whatever. A number of factors have been shown to modulate macrophage pinocytosis (5), but the available data permit few inferences as to the nature of the nonimmunologic cell surface receptors. Proteolytic enzymes (6), neuraminidase (7, 8) and RNase (9) have been shown to modify the properties of mammalian cell surfaces. Vaughan (10) and Rabinovitch (11) have observed that trypsin treatment of macrophages decreased the attachment of effete and aldehyde-treated red cells to the macrophage surface, while Allen and Cook (12) have shown a similar effect of proteolytic enzymes on the attachment of opsonized bacteria. It was therefore anticipated that an analysis of the effects of the several enzymes on pinocytosis might provide information on the molecular character of macrophage receptors. In this first study, uptake of protein was assessed rather than binding.

Materials and Methods. Macrophages were harvested from peritoneal cavities of male Sprague-Dawley rats (Charles River Inc.) in heparinized Hanks' salt solution. In a few experiments freshly isolated cells were incubated in 10-ml Erlenmeyer flasks at 37° on a shaker in the presence of fluorescein-labeled, aggregated albumin. The cells were subsequently plated on coverslips for microscopic examination. In most experiments, cells from the peritoneal cavity were cultured on coverslips in 21 × 70-mm glass shell vials fitted with Morton stainless steel tube closures (Bellco Glass Inc., Vineland, N.J.). Waymouth's medium with 20% fetal calf serum was used, and the cultures were maintained in a 5% CO_2 in air environment at 37°. The cells were cultured for 2 hr, and those cells that did not adhere were removed by repeated washing. More than 95% of the cells remaining on the coverslips under these conditions were mononuclear phagocytes.

Cells were exposed to enzymes for a 30-min period; they were then washed in three changes of medium before testing for pinocytotic activity or further maintenance *in vitro*. Cells were tested for pinocytotic activity by exposing them to aggregated albumin labeled with fluorescein (Fl-Agg Alb) (13) or fluorescein-labeled ferritin (Fl-Ferritin) in Waymouth's medium, usually for 1 or 2 hr. The coverslips were washed with three changes of Hanks' solution and one change of 0.15 *M* ammonium acetate; they were then removed from their vials and dried overnight at room temperature. The coverslips were mounted in very low fluorescence

[1] Supported by U.S. Public Health Service Grants AM-08516 and HE-03174.

immersion oil (Cargill) for examination in the fluorescence microscope or stained for ferric iron by the ferrocyanide procedure and counterstained with nuclear fast red.

At least 100 cells on 2 coverslips were scored in each group in an experiment. Cells were rated high uptake (++), low uptake (+), or no uptake (0). The treatment of the cells being scored was not known to the observer. All cells in an experiment were scored by a single observer.

Protein synthesis was measured by the incorporation of leucine-^{3}H (New England Nuclear Corp.) into TCA-insoluble fraction. Cells were exposed to leucine-^{3}H incorporated into Waymouth's medium lacking additional leucine for 30 min. Cells on coverslips were washed 3 times in Hanks' salt solution, 2% PCA, ethanol 2 times, air-dried, and counted in toluene-based solution in a liquid scintillation counter (Packard Tri-Carb).

Cycloheximide was purchased from Nutritional Biochemical Corporation. Aggregated human albumin was purchased from E. R. Squibb and Sons, New Brunswick, N.J. as *Albumotope Carrier*. Ferritin and human albumin were purchased from Pentex Inc., Kankakee, Ill. Chymotrypsin, trypsin, and RNase I were purchased from Worthington Biochemical Corp., Freehold, N.J. and neuraminidase from Sigma Chemical Co., St. Louis, Mo. Pronase was purchased from Calbiochem, Los Angeles, Calif. The proteins were labeled with fluorescein isothiocyanate (FITC) at pH 8.5 in 0.1 *M* sodium bicarbonate at room temperature. The ratio of FITC to protein used was 1:20 mg/mg. Unbound fluorescein was removed by one or more passes over Sephadex G-25 in 0.85% saline, pH 7.4. Labeled protein was concentrated as required by ultrafiltration in collodion membranes.

Chymotrypsin was inactivated with diisopropylfluorophosphate, dialyzed thoroughly, and concentrated as required by ultrafiltration or lyophilization. The preparation used

TABLE I. Effect of Enzyme Treatment on Macrophage Pinocytosis.

Treatment	No. of cells scored	Cells exhibiting pinocytosis (%) ++	+	0
A[a]				
Pronase (1 mg/ml)	100	4	21	75
Chymotrypsin (1 mg/ml)	100	4	49	48
Trypsin (1 mg/ml)	100	8	38	53
Control	100	48	34	8
B[b]	No. of expts.			
Chymotrypsin (1 mg/ml × 30 min)	3	7.0 ± 3.5[c]	19.0 ± 10.8	77.0 ± 9.1
Albumin (human) (1 mg/ml × 30 min)	3	82.0 ± 12.0	8.0 ± 10.2	10.0 ± 2.9
Control	4	94.2 ± 3.1	1.2 ± 1.5	4.5 ± 2.6
C[d]				
Neuraminidase (1 mg/ml × 30 min)	3	92.0 ± 2.6	1.7 ± 7.5	6.3 ± 2.1
RNase (1 mg/ml × 30 min)	4	88.8 ± 10.7	3.8 ± 2.6	7.5 ± 3.1
Chymotrypsin (1 mg/ml × 30 min)	4	4.0 ± 4.8	7.0 ± 4.7	89.0 ± 1.4
Albumin (human) (1 mg/ml × 30 min)	3	96.3 ± 3.2	0.7 ± 0.6	3.0 ± 2.6
Control	6	96.5 ± 2.3	1.2 ± 1.9	2.3 ± 1.6

[a] Macrophages were freshly harvested, exposed to enzyme in shaking flasks for 30 min, and then to Fl-Agg-Alb in shaking flasks for 2 hr. After washing, the cells were plated on coverslips and prepared for viewing.

[b] Macrophages were cultured on coverslips overnight before exposure to enzyme. Fl-Agg-Alb was used as the test protein. At least 200 cells were scored in each experiment.

[c] (± 1 SD).

[d] Macrophages were handled as in Part B, but Fl-ferritin was used as the test protein. At least 200 cells were scored in each experiment.

TABLE II. Comparison of the Effect of Active and DFP-Inhibited Chymotrypsin on Macrophage Pinocytosis.[a]

Treatment	No. of coverslips scored	Cells exhibiting pinocytosis (% ± 1 SD)					
		Fl-Agg-A1b			Fl-Ferritin		
		++	+	0	++	+	0
Chymotrypsin (1 mg/ml × 30 min)	3	7.0 ± 3.5	19.0 ± 10.8	74.0 ± 9.1	1.3 ± 0.6	11.3 ± 1.5	87.0 ± 1.0
DFP-Chymotrypsin (1 mg/ml × 30 min)	3	93.2 ± 3.2	3.0 ± 2.2	3.8 ± 1.3	94.3 ± 5.5	1.7 ± 1.5	4.3 ± 4.5
Control	3	94.2 ± 3.1	1.2 ± 1.5	4.5 ± 2.6	89.5 ± 5.0	2.7 ± 1.5	8.0 ± 6.6

[a] Macrophages were cultured overnight before exposure to enzymes. Cells were washed free of enzyme and exposed to labeled protein for 2 hrs. One hundred cells were scored on each coverslip.

had less than 1% of the initial hydrolytic activity of chymotrypsin, tested with acetyl tyrosine ethyl ester as substrate.

Results. Pronase, chymotrypsin, trypsin, RNase, and neuraminidase were tested for inhibitory effects on the uptake of aggregated albumin and ferritin; human albumin was used as a nonenzyme protein control. The results in Table I indicate that the proteolytic enzymes markedly inhibited protein ingestion while RNase, neuraminidase, and albumin were inactive.

Treatment of macrophages with pronase before they were allowed to attach to glass led to a considerable reduction in their ability to adhere. Chymotrypsin, at 1 mg/ml, effectively inhibited pinocytosis, but did not prevent cells from subsequently adhering to glass, and treatment of macrophages already attached to glass with chymotrypsin did not remove any appreciable number of cells. Fluorescein-labeled chymotrypsin itself was not taken up by the cells. A requirement for active proteolytic enzyme was demonstrated by the ineffectiveness of DFP-chymotrypsin (Table II). Macrophages after exposure to chymotrypsin exhibited normal impermeability to lissamine green, and protein synthesis was unaffected by treatment with chymotrypsin.

To investigate their capacity to recover from the inhibition of pinocytosis induced by chymotrypsin, macrophages treated with enzyme were subsequently maintained in culture. The cells regained a large part of their pinocytotic capability within 6 hr (Table III). Two possible sources of the protease-sensitive component required for pinocytosis were considered: (i) serum, and (ii) macrophage synthetic activity. It was not possible to remove all serum from the macrophages during the recovery period, but recovery of pinocytotic capability was similar in 1% serum and 20% serum (Table IV).

In order to further define the source of the protease-sensitive component required for the pinocytosis of protein, protein synthetic activity of macrophages was inhibited with cycloheximide during the recovery period. Exposure of macrophages to 5 μg/ml of cycloheximide inhibited the incorporation of

TABLE III. Recovery of Macrophage Pinocytosis After Chymotrypsin Treatment.[a]

Treatment	No. of cells	Cells exhibiting pinocytosis (%) ++	+	0
Control (no chymotrypsin)	100	83	8	9
Chymotrypsin (no recovery) (1 mg/ml × 30 min)	100	0	0	100
Chymotrypsin (1 mg/ml × 30 min) recovery,[b] 2 hr	100	0	8	92
4 hr	100	12	66	22
6 hr	100	74	17	9

[a] Macrophages were cultured overnight before exposure to chymotrypsin.

[b] Recovery refers to culture of macrophages in 20% serum after chymotrypsin treatment, prior to testing for pinocytosis. Test protein was Fl-aggregated albumin to which the cells were exposed for 2 hr.

leucine-^{3}H into the trichloroacetic acid insoluble material 62 ± 10%. Doubling the concentration of cycloheximide did not further inhibit leucine incorporation. When cells were treated with chymotrypsin and then exposed to cycloheximide, there was no enhancement of the inhibition of protein synthesis by cycloheximide.

When tested for its effect on ingestion of protein (Table V), cycloheximide had a small inhibitory effect (Table VD). When macrophages were continuously exposed to cycloheximide following treatment with chymotrypsin, the cells failed to recover the capacity to ingest protein (Table VC).

Discussion. The results indicate the existence of a factor required for the ingestion of protein that is susceptible to proteolytic enzymes. Location of this factor at the cell surface is suggested by the observation that under those conditions in which chymotrypsin is effective the enzyme itself is not taken into the cell. Previous experiments have demonstrated that chymotrypsin can affect strictly surface properties of cells, such as electrophoretic mobility (7) and cell adhesion (6). The possibility that proteolytic enzymes primarily interfere with some general cell function and only secondarily with pinocytosis is unlikely in view of the maintenance of both protein synthesis and cell membrane selective permeability in cells treated with chymotrypsin.

Since the experiments assessed pinocytotic activity rather than binding, the results are not directly interpretable in terms of an

TABLE IV. Effect of Serum Concentration on Recovery of Pinocytosis by Macrophages After Chymotrypsin Treatment.[a]

Treatment	Recovery	No. of coverslips scored	Cells exhibiting pinocytosis (% ± 1 SD) ++	+	0
Chymotrypsin	None	3	0	0.3 ± 0.6	99.7 ± 0.6
	6 hr in 20% serum	2	76.0	5.5	19.0
	6 hr in 1% serum	3	64.3 ± 4.2	6.3 ± 6.5	29.4 ± 4.7
DFP-Chymotrypsin	None	3	97.0 ± 2.6	0	2.7 ± 2.1

[a] Conditions were the same as those described in Table III.

TABLE V. Effect of Inhibition of Protein Synthesis on Recovery of Pinocytosis.[a]

Initial treatment	Conditions of 3.5 hr recovery	No. of expts.	Cells exhibiting pinocytosis (% ± 1 SD) ++	+	0
A Chymotrypsin (1 mg/ml × 30 min)	No recovery period	4	1.8 ± 1.7	6.5 ± 4.5	91.8 ± 4.8
B	Control medium	4	49.0 ± 22.4	5.8 ± 3.2	45.2 ± 22.9
C	Cycloheximide	4	0.5 ± 0.6	7.8 ± 5.1	91.8 ± 5.5
D Control medium × 30 min	Cycloheximide	4	66.0 ± 8.4	9.5 ± 7.8	24.5 ± 10.8
E	Control medium	4	92.2 ± 1.5	1.2 ± 1.0	6.5 ± 1.3

[a] Conditions were the same as those described in Table III; p values for chance occurrence of differences in mean values between (++) groups calculated by t test are as follows: A–B, $< .01$; B–C, $< .01$; C–D, $< .01$; D–E, $< .01$.

effect on surface receptors. It is conceivable that a cell surface component involved in ingestion rather than binding is attacked. From other experiments in which binding has been examined directly, it is evident that proteolytic enzymes are capable of destroying receptors (11, 12, 14–17). However it should be noted that not all receptors are susceptible to the action of proteolytic enzymes (16–20).

The slow recovery of pinocytosis after exposure of macrophages to chymotrypsin and the inhibition of recovery by cycloheximide favor the argument that the factor sensitive to proteolytic enzymes is generated by the macrophage rather than adsorbed from serum. Such a conclusion is counter to that of Vaughan (10) who studied the recovery of the ability of macrophages to bind effete, autochthonous red blood cells after protease treatment and concluded that the factor sensitive to trypsin was derived from serum. Since in the present set of experiments recovery was not achieved in the absence of serum, a contribution of serum to the protease-sensitive factor cannot be excluded; the results do nonetheless indicate a requirement for protein synthesis by the macrophage during recovery.

Recovery of functional properties of cells following destruction by proteolytic enzymes has been reported for several cell types (21–23). In the two instances in which the experiments included interference with protein synthesis, recovery was inhibited as in the case of macrophage pinocytosis (21, 23).

Summary. Macrophages exposed to chymotrypsin, trypsin, or pronase failed to ingest aggregated albumin or ferritin *in vitro*. Treatment with neuraminidase, RNase, albumin, or DFP-chymotrypsin had no apparent effect on macrophage pinocytosis. When cells treated with active chymotrypsin were subsequently maintained in culture, they recovered their ability to ingest protein. This recovery was inhibited by partial inhibition of protein synthesis with cycloheximide. Since chymotrypsin itself is not ingested, the results are interpreted to indicate the removal or inactivation of a surface factor required for pinocytosis that is generated by the cell.

Mrs. LeMoyne Mueller and Miss Pamela Pritzl have provided excellent technical assistance for these experiments.

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Received Oct. 23, 1970. P.S.E.B.M., 1971, Vol. 138.