ENERGY DEPENDENT ENDOCYTOSIS BY ERYTHROCYTE GHOSTS

V. EFFECTS OF NEURAMINIDASE AND PRONASE DIGESTION

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SUMMARY: Mild treatment of washed erythrocytes with pronase or of erythrocyte ghosts with neuraminidase led to removal of a few well defined components of the membrane, those which were exposed on the outer face of the membrane. Such digestion had no effect on the ATPase activity or upon energy dependent endocytosis as carried out by ghosts isolated from cells which had been digested. Thus, the protein components of the machinery of endocytosis are inaccessible to the pronase enzymes from the outside of the intact erythrocytes. Treatment of erythrocytes with high levels of pronase led to a much more extensive non-specific proteolysis which inactivated both ATPase and energy-dependent endocytosis.

INTRODUCTION: Endocytosis, or vacuole intake, by erythrocyte ghosts has been observed in several laboratories (1-3). When carried out under isotonic conditions it requires ATP and Mg^{++} (4). In order to study the mechanism by which this energy dependent endocytosis occurs, some of the components of the erythrocyte membrane were removed by methods which did not destroy the morphology of that membrane and the effect of this removal on ATP hydrolysis and on endocytosis was studied. When whole red cells are digested with enzymes and ghosts subsequently prepared, a few well-defined components are partially digested (5-9). These studies have provided a relatively well-defined tool for investigation of the effects of removal of these components upon energy dependent endocytosis.

EXPERIMENTAL METHODS: Erythrocytes and erythrocyte ghosts were obtained from three week old human blood from the North Carolina Memorial Hospital Blood

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Bank, using the preparative method previously described (10). For digestion by pronase, pronase isolated from Streptomyces griseus was obtained from Sigma Chemical Company, St. Louis, Missouri. After the standard washing, in phosphate buffer, the whole erythrocytes were suspended to 20% hematocrit in the isotonic phosphate buffer. A concentrated aqueous solution of pronase was added to the cell suspension in order to obtain the desired final concentration of proteolytic enzymes. Incubation was then carried out at 37°C with constant stirring for 60 min. The digestion was stopped by addition of 2 to 3 volumes of cold phosphate buffer and the cells were centrifuged and washed 3 more times with phosphate buffer before hemolysis. Pronase digestion of ghosts was carried out in 50 mM Tris-HCl pH at 37°C, 7.4 containing 5 mM Mg Cl_2. The membrane protein concentration was 1 mg per ml, which gave approximately the same number of cells per ml as were present in the digestion of the whole cells, calculated using the data of Dodge, et. al. (11). The incubation was carried out for 10 min and was stopped and the ghosts washed in a similar manner as described for whole erythrocytes, except that the Tris buffer was utilized for the washes of ghosts.

Neuraminidase, type VI from Cl. perfringens with an activity of about two units/mg was obtained from Sigma. Treatment was carried out in an aqueous medium containing 2 mg/ml of ghost protein and 0.05-0.1 unit/ml of neuraminidase, and which was 5 mM in Mg Cl_2. The buffer was piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) neutralized with triethanolamine and was present at a concentration of 50 mM and a pH of 6.5. The mixture was incubated 30 min at 37°C and washed two times with the same cold buffer, and once with a pH 7.4 buffer.

After digestion, if electrophoresis was to be carried out, the ghosts were solubilized in the detergent-buffer solution and were immediately brought to boiling for 3 min in order to halt proteolytic digestion.

The methods utilized for measurement of ATPase, endocytosis, and phospholipid were described previously (10). Sialic acid was measured by the method

TABLE I

EFFECTS OF NEURAMINIDASE TREATMENT OF GHOSTS

Activities :	*Undigested*	*Digested*
Endocytosis (%)	100	100
ATPase (nmoles, mg^{-1}, min^{-1})		
Mg^{++}	4.0	3.8
Mg^{++}, Na^{+}, K^{+}	8.7	8.0
Mg^{++}, Ca^{++}	15.0	14.5
Components :		
Sialic Acid (nmoles, mg^{-1})	89	18
Phospholipid/Protein (w/w)	0.42	0.43
Total Hexose (% of Untreated)	100	70

of Warren (12) after acid hydrolysis in 0.5 N sulfuric acid at 80°C for 60 min. Total hexose was assayed by the method of Dubois et. al. (13) and gel electrophoresis was carried out according to the method of Neville (14).

RESULTS: As expected, neuraminidase digestion proved to be the mildest treatment used, and had no effect on ghost morphology as observed by phase contrast light microscopy or by electron microscopy. Table I shows that neuraminidase digestion of ghosts removed almost all of the sialic acid from the membranes, but that it caused no difference in the level of the ATPase nor did if affect energy dependent endocytosis.

The effects of pronase digestion were more extensive. Ghosts prepared from whole erythrocytes which had been digested with from 1 microgram per ml to 100 micrograms per ml of pronase showed no morphological changes. Ghosts prepared from whole erythrocytes treated with one mg per ml of pronase looked normal but the membranes tended to retain hemoglobin; even 7 washes failed to produce white ghosts from these cells. Incubation of ghosts prepared from

the cells treated with this highest level of pronase led to breakdown of the intact ghosts into small membrane vesicles after 30 min.

When ghosts were treated directly with pronase, a much more extensive digestion of membrane occurred at very low levels of added pronase. Ghosts digested with one microgram of pronase per ml disintegrated into small vesicles upon incubation after washing, just as did the ghosts prepared from whole cells which had been treated with a 1000-fold higher level of pronase. Ghosts which were treated with 10 microgram per ml of pronase disintegrated into small vesicles immediately, without further incubation, indicating extensive damage to the membrane.

TABLE II

EFFECT OF PRONASE DIGESTION OF ENDOCYTOSIS AND ATPASE[a]

	ATPase activity (nmoles, mg^{-1}, min^{-1})			*Endocytosis (%)*
Ghosts made from whole cells digested by	Mg^{++}	Mg^{++}, Na^{+}, K^{+}	Ca, Mg^{++}	
none	4.0	8.7	15.0	100
1 µg/ml	4.0	8.7	13.0	100
10 µg/ml	4.0	7.8	13.0	100
100 µg/ml	3.3	6.0	12.3	90
1 mg/ml	2	3	5	0[b]
Ghosts digested by :				
1 µg/ml	2	2	3	0[b]
10 µg/ml	0	0	0	0[c]

a. The data shown were taken from a typical experiment; this type of experiment was repeated more than five times.

b. Ghosts appeared normal after preparation, but disintegrated into small vesicles during the endocytosis assay. This may be due to additional digestion by residual membrane-bound pronase.

c. After pronase digestion, the ghosts had completely disintegrated into small vesicles.

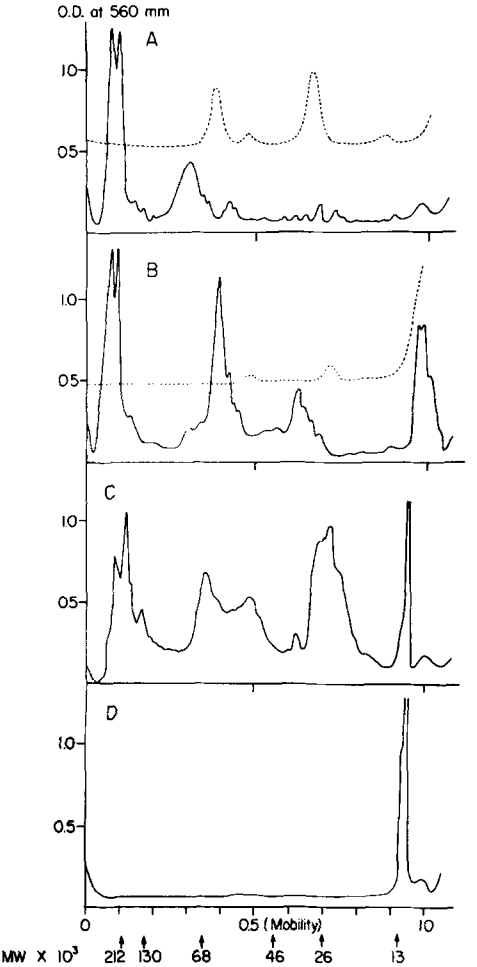

Figure 1: Effect of Pronase Digestion on Subunit Size of Membrane Proteins. The traces represent absorbance versus distance from the origin; solid lines show gels stained for protein, dashed lines, gels stained for carbohydrate. 1A shows the pattern normally obtained from untreated human erythrocyte ghosts; 1B shows the effect of digestion of whole cells with 100 μg of pronase; 1C shows the effect of digestion of ghosts with 1 μg/ml of pronase and 1D shows the effect of digestion of ghosts with 10 μg/ml of pronase.

The effects of pronase digestion on the ATPase activity and on endocytosis are shown in Table II. Digestion of whole erythrocytes with low levels of pronase had no effect on ATPase or endocytosis. When the pronase concentration was raised to 100 micrograms per ml, a small inhibition of both ATPase and endocytosis began to appear, while digestion with yet higher levels of pronase inhibited the ATPase drastically and made it impossible to assay

TABLE III

EFFECT OF PRONASE DIGESTION ON SIALIC ACID, HEXOSE AND PHOSPHOLIPID CONTENT OF GHOSTS

	Sialic Acid (*nmoles, mg^{-1}*)	*Hexose* (*% of untreated*)	*Phospholipid/ Protein* (*w/w*)
Ghosts made from whole cells digested by :			
none	89	100	0.42
1 µg	48	70	0.43
10 µg	35	65	0.43
100 µg	21	55	0.45
1 mg	20	54	0.48
Ghosts digested by :			
1 µg	45	60	0.64
10 µg	21	50	0.70

for endocytosis because of breakdown of the ghost membrane. The effects of the same treatments on the subunit molecular weights of the erythrocyte membrane proteins are shown in Figure 1. This figure shows that those treatments which caused extensive digestion of the proteins normally inaccesible to pronase also inactivated ATPase and endocytosis.

The effect of these various pronase treatments upon the composition of the resulting ghosts is shown in Table III. The higher phospholipid content after more drastic digestion suggested that substantial amounts of protein were removed from the ghosts by these treatments.

DISCUSSION: Ghosts lacking in the two external protein components behaved normally in carrying out energy dependent endocytosis. Thus, it is clear that none of the essential components of the system responsible for endocytosis had been damaged by digestion of the externally excessible proteins. We may conclude that the machinery of energy dependent endocytosis, so far as this

endocytosis depends on the mediation of proteins, is inaccessible from the outside of the intact erythrocyte.

In all probability, this indicates that the protein components of endocytosis are either embedded in the membrane or lie in the inner face of the membrane, inaccessible to outside reagents.

Digestion of whole erythrocytes by higher levels of pronase, or digestion of ghosts with low levels of pronase, caused far more extensive alterations in the erythrocyte membrane. Under these conditions, both ATPase and endocytosis were concurrently inactivated. This reinforces previous observations concerning the requirement of endocytosis for the presence of an active ATPase (4, 10).

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