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Immunochemical Studies of Infectious Mononucleosis
IV. Effect of Proteases on the Glycoprotein of Horse Erythrocytes[1] (37961)

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Horse erythrocytes possess a membrane-bound receptor for the heterophile antibody of infection mononucleosis (1). Recently, we have described the isolation of this receptor and its characterization as a sialoglycoprotein (2). We have also presented evidence that this antigen is the predominant glycoprotein of horse erythrocytes (3). In this regard, as well as gross biochemical analysis, it bears close analogy to the MN glycoprotein of human erythrocytes, although the latter appears, on sodium dodecyl sulfate polyacrylamide gel electrophoresis, to be of larger molecular size (3).

The major glycoprotein of human erythrocytes has been extensively studied. It is known to be exposed on the exterior of the membrane surface, perhaps even spanning the lipid bilayer (4, 5). This glycoprotein in the red cell is susceptible to cleavage by trypsin (6), pronase (7), and papain (8). The fragments released by proteases retain receptor activity for M or N antibodies (6) and phytohemagglutinin (9).

Much less information is available concerning the effect of proteases on the glycoproteins of other mammalian erythrocytes. The present studies were undertaken to study the effect of three proteolytic enzymes, trypsin, papain, and pronase, on the principal glycoprotein of horse erythrocytes, the infectious mononucleosis receptor. This effect was investigated by three approaches: release of sialic acid and other carbohydrates from the cells; fate of the major periodic acid Schiff staining band in sodium dodecyl sulfate polyacrylamide gel electrophoresis of stroma derived from treated erythrocytes; and activity of the membrane infectious mononucleosis receptor with antibody.

Materials and Methods. Erythrocytes. Blood from individual horses was collected asceptically by venipuncture into sterile Alsever's solution and stored at 4°. Prior to enzyme digestion, the erythrocytes were separated from plasma and buffy coat by centrifugation at 3000*g* for 20 min and washed thrice in saline (0.85%) buffered with 0.005 *M* sodium phosphate, pH 7.4 (phosphate-buffered saline).

Sheep blood for hemagglutination inhibition tests was obtained from Cordis Laboratories.

Enzyme digestions. Trypsin (2× crystalline, Sigma) in 0.041 *M* Tris-buffered saline, pH 8.1, containing 0.015 *M* $CaCl_2$, pronase (B grade, Sigma) in phosphate-buffered saline, or papain (2× crystalline, Sigma) in saline containing 0.006 *M* mercaptoethanol, 0.005 *M* cysteine, and 0.001 *M* ethylene diaminetetraacetic acid was incubated with washed, packed horse erythrocytes at an enzyme to cell ratio of 0.25 mg/ml for 1 hr at 37° with gentle rotation.

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Controls consisted of cells incubated in the three enzyme diluents without enzyme. The incubation mixtures were then centrifuged at 4°. The supernatants were either frozen at −20° or added to one-fourth volume of 25% trichloroacetic acid. After centrifugation of 10,000*g* for 30 min at 4°, the trichloroacetic acid-soluble fraction was neutralized with sodium hydroxide, dialyzed against 2 changes of distilled water, and frozen at −20°. The enzyme-treated erythrocytes were washed thrice with phosphate-buffered saline. A portion of treated cells were reserved for hemagglutination inhibition studies and the residual used to prepare hemoglobin-free ghosts as previously described (2).

Analytical procedures. Sialic acids were assayed by the thiobarbituric acid method (10) using *N*-acetylneuraminic acid (grade III, Sigma) as standard. Samples were hydrolyzed in 0.1 *N* H_2SO_4 at 80° for 1 hr before analysis. Since the predominant sialic acid in horse erythrocytes is the *N*-glycolyl derivative (2, 11), values obtained were corrected for the difference in molecular extinction coefficients of *N*-acetyl and *N*-glycolylneuraminic acids (10). Neutral carbohydrates and amino sugars were determined as alditol acetates by gas–liquid chromatography as described by Griggs *et al.* (12). The column used was 3% ECNSS on Gas-ChromQ 100/120 mesh (Regis Chemical Co.). The internal standard, perseitol, was added to each sample prior to acid hydrolysis. Standard curves were prepared for each sugar in which molar ratios of the sugar to perseitol were compared to the peak area ratios. The molar response factors calculated from the slopes of these curves were then used to estimate monosaccaride concentrations in the unknown samples as suggested by Clamp *et al.* (13).

Serologic tests. Sera were obtained from patients with clinically confirmed heterophile antibody positive infectious mononucleosis. The serum was heated at 56° for 20 min. Hemagglutination inhibition tests were done as previously described (2) using, as inhibitor, stroma dispersed in phosphate-buffered saline by two cycles of freezing and thawing followed by ultrasound treatment for 5 min. Activities of inhibition were expressed as the concentration of stroma in μg/ml which completely inhibited the agglutination of sheep erythrocytes by four hemagglutinating doses of infectious mononucleosis serum. Hemagglutination titers of treated horse erythrocytes were determined using a purified antibody preparation. The infectious mononucleosis antibody was isolated from serum by absorption to glutaldehyde-polymerized glycoprotein from horse erythrocytes. After washing, the heterophile antibody was eluted with 3 *M* $MgCl_2$ (14). An equal volume of 0.5% suspension of horse erythrocytes was added to serial dilutions of purified antibody. After incubation for 2 hr at 22°, the endpoint of hemagglutination was determined with a hand lens.

Polyacrylamide gel electrophoreses. Erythrocyte membranes were fractioned by electrophoresis on 7.5% polyacrylamide

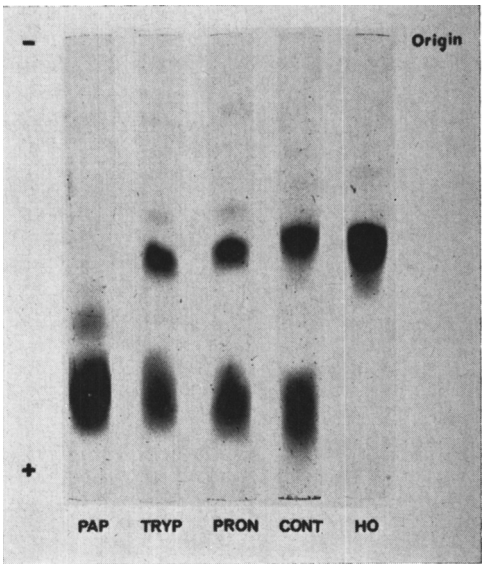

FIG. 1. Electrophorograms of stroma from protease-treated and untreated horse erythrocytes and of purified horse erythrocyte glycoprotein on 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Stained with periodic acid-Schiff reagent. PAP = papain treated; TRYP = trypsin treated; PRON = pronase treated; CONT = phosphate-buffered-saline treated; HO = purified glycoprotein from horse erythrocyte.

gels in the presence of 0.1% sodium dodecyl sulfate as previously described (3).

Results. As illustrated in Fig. 1, papain was the only one of the three proteases tested which produced a visible effect on the mobility in sodium dodecyl sulfate polyacrylamide gel electrophoresis of the major glycoprotein in horse erythrocyte membranes. Stroma prepared from trypsin- or pronase-treated erythrocytes had a major periodic acid-Schiff staining band of mobility identical to that of stroma from untreated erythrocytes and to that of purified infectious mononucleosis receptor glycoprotein isolated from horse erythrocytes (3). The stroma prepared from papain-treated erythrocytes showed a much fainter band of faster mobility. The major glycoprotein band was not visible. Protease treatments were without effect on the dense band of periodic acid-Schiff staining material which migrated with the dye front and is thought to represent membrane lipid (15). The patterns obtained when the gels were stained with Coomassie blue (Fig. 2) were not detectably different for stroma derived from papain- or pronase-treated erythrocytes as compared to control stroma. However, trypsin treatment appeared to result in the loss of some of the peptide bands seen in the other three gels.

As shown in Table I, papain was the most effective of the three enzymes in removing sialic acid from the cell surface. The amount released was similar to that reported released from human red cells by trypsin (6). However, trypsin was completely ineffective in removing sialic acid

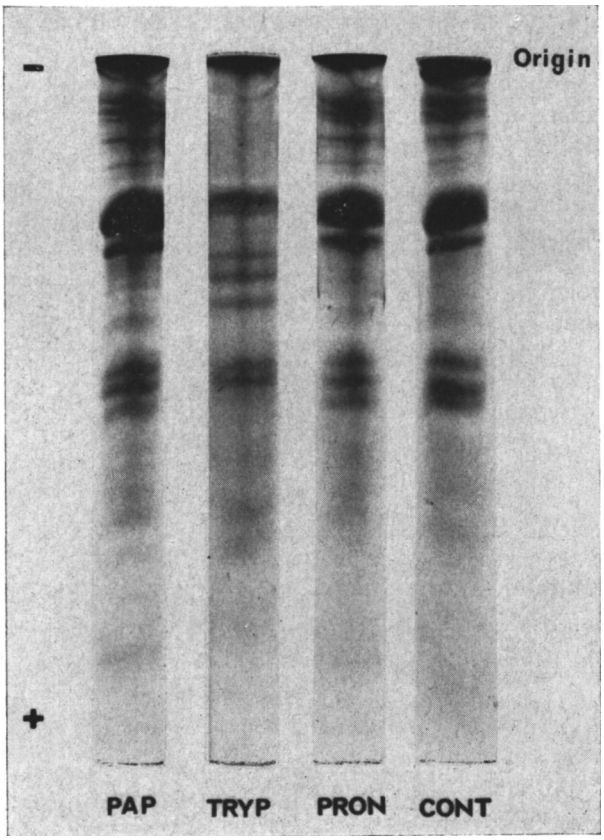

FIG. 2. Electrophorograms of stroma from protease-treated and untreated horse erythrocytes on 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Stained with Coomassie blue. Symbols are given in Fig. 1.

TABLE I. Release of Sialic Acid from Horse Erythrocytes by Proteolytic Enzymes.

Treatment of erythrocytes	nmoles Sialic acid released/ml packed cells
Papain	204.7
Followed by pronase	0.6
Followed by trypsin	0.8
Pronase	94.3
Followed by papain	104.7
Trypsin	1.6
Control	1.0

from horse erythrocytes. Pronase treatment released only one-half as much sialic acid as did papain. This fraction accessible to pronase was obviously a portion of that accessible to papain. No additional sialic acid was released by pronase from cells previously treated by papain. Also, pretreatment of cells by pronase reduced the amount of sialic acid cleaved by papain. After dialysis and trichloroacetic acid precipitation of the supernatant from papain-treated cells, over 90% of the released sialic acid was recovered in the nondialyzable, trichloroacetic acid-soluble fraction, indicating the glycopeptide nature of the cleavage product. Sialic acid content of stroma from papain-treated erythrocytes was only 50% of that in stroma from control erythrocytes. The papain supernatant was examined for the presence of other sugars and the results are presented in Table II. Sialic acid was the most abundant constituent released by papain. Galactose was the predominant neutral sugar with a molar ratio over mannose of 6:1. Twice as much *N*-acetylgalactosamine was present as was *N*-acetylglucosamine. Molar ratio of hexose:hexosamine:sialic acid was 1:1:1.3. Small quantities of fucose were also released.

TABLE II. Carbohydrate Composition of the Crude Glycopeptide[a] Released from Horse Erythrocytes by Papain.

Component	nmoles/ml packed cells
Galactose	127.5
Mannose	21.6
Fucose	13.1
N-Acetylgalactosamine	110.2
N-Acetylglucosamine	51.4
N-Glycolylneuraminic acid	190.0

[a] The trichloroacetic acid-soluble, nondialyzable fraction.

Table III shows the effect of the three proteases tested on the agglutination of horse erythrocytes by heterophile antibody of infectious mononucleosis. The antibody used for this test was purified from serum in order to eliminate the other specificities of antibody present in human sera which also react with horse erythrocytes (16). In relation to the control, the cells treated with papain show a twofold reduction in titer, whereas those treated with either trypsin or pronase are seen to have a twofold increase.

The ability of stroma prepared from protease-treated erythrocytes in inhibiting the agglutination of sheep erythrocytes by serum from a patient with infectious mononucleosis is shown in Table IV. At the highest concentration tested, 2000 μg/ml, stroma from papain-treated erythrocytes did not inhibit. Stroma from pronase- or trypsin-treated cells were effective inhibitors at concentrations 10–20 times less and were, in fact, 2–4 times as effective as the stroma from control erythrocytes.

Discussion. Detailed knowledge of the complex carbohydrates present on mammalian cell membranes is limited to only a few cell types. Perhaps the most intensely studied is the major glycoprotein of the human erythrocyte membrane which has been suggested by Winzler as a prototype (7). Our previous studies (2, 3) have

TABLE III. Effect of Proteases on Hemagglutination of Horse Erythrocytes by Purified Heterophile Antibody[a] of Infectious Mononucleosis.

Treatment of cells	Reciprocal titer
Phosphate-buffered saline	32,000
Papain	16,000
Trypsin	64,000
Pronase	64,000

[a] Purified antibody isolated from serum of patient with infectious mononucleosis by an immunoabsorbent containing the heterophile glycoprotein antigen of beef erythrocytes.

TABLE IV. Hemagglutination Inhibition Activity of Stroma from Protease-treated Horse Erythrocytes.

Treatment of erythrocytes	μg/ml stroma inhibiting four hemagglutinating doses of infectious mononucleosis serum[a]
Phosphate-buffered saline	480
Papain	>2000
Trypsin	120
Pronase	240

[a] Tested with sheep erythrocytes.

shown points of similarity as well as difference between the human erythrocyte glycoprotein and that of the horse erythrocyte. Both may be obtained in soluble form and in large part free from other membrane constituents by the same extraction procedure. In terms of gross biochemical analysis, these purified glycoproteins are similar. The soluble glycoprotein fractions from each species show a major band on polyacrylamide electrophoresis which corresponds in mobility to the major glycoprotein of the unextracted membrane dissolved directly in sodium dodecyl sulfate. However, the apparent molecular weight of the glycoprotein in human erythrocytes is larger than that in the horse erythrocyte. Both glycoproteins have antigenic activity associated with terminal sialic acid residues. However, the glycoprotein from human erythrocytes carries blood-group MN activity and that from horse erythrocytes reacts with Paul–Bunnell heterophile antibody of infectious mononucleosis.

In the present studies, intact horse erythrocytes were treated with proteases: one of limited substrate specificity, trypsin, and two of broad specificity, pronase and papain. All three enzymes are known to release glycopeptides from human erythrocytes (6–8). We found that the major glycoprotein of horse erythrocytes was not cleaved by trypsin and only a portion was susceptible to pronase action. Papain appeared to cleave a larger proportion of the glycoprotein molecules as evidenced by the disappearance of the major periodic acid-Schiff staining band on polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Apparently, a residual smaller molecular weight fragment which still carried some of the carbohydrate constituents was left attached to the membrane, since a fast-moving band which stained with periodic acid-Schiff was observed. This finding is similar to results obtained by treating human erythrocytes with trypsin (18, 19). The resistance of the membrane glycoprotein to the action of trypsin may be interpreted as an absence of accessible lysine or arginine residues (20). Its partial resistance to the action of pronase, a mixture of endo- and exopeptidases, may indicate heterogeneity of arrangement of the glycoprotein on the membrane surface, as suggested by Uhlenbruck and Wintzer regarding the erythrocytes of several species (21).

Analysis of the carbohydrates of crude TCA-soluble, nondialyzable fraction of the papain digestion of horse erythrocytes also indicated that the major glycoprotein of the membrane was cleaved. The papain fragments and the intact glycoprotein (2) contained the same constituent carbohydrates and in the same order of relative proportion. Sialic acid was the most abundant component followed in descending order by galactose, *N*-acetylgalactosamine, mannose, and fucose. Molar ratios of hexose:hexosamine:sialic acid for both of these materials was close to unity. The proportions of carbohydrate are very similar to those reported for trypsin fragments from human erythrocytes (6). This glycopeptide is known to contain several types of complex oligosaccharide side chains (17). It is likely that the glycoprotein from horse erythrocytes will share similar complexity. Studies are presently underway to further purify and characterize the papain fragments from this species.

The amount of membrane sialic acid lost from horse erythrocytes treated by papain is about the same as that from horse cells treated with neuraminidase (11). A substantial fraction of horse-erythrocyte sialic acid is known to be bound to glycolipid (22) and this may be neuraminidase resistant. At least a portion of the sialic acid not cleaved by papain or

neuraminidase is in this fraction. Although papain-treated cells have reduced titer against infectious mononucleosis antibody, much activity was still present. Some activity may be associated with glycolipid, with a fraction of glycoprotein inaccessible to papain, or with papain fragments which remain associated with the membrane. It is impossible to estimate the actual amount from these studies since the cleavage of a portion of negatively charged sialic acid would accentuate agglutination of the cells with antibody (23). In fact, Lee *et al.* have reported papain to be without effect on the infectious mononucleosis receptor of horse cells (16). These workers, however, did not use specifically purified antibody and possibly the loss of a portion of heterophile receptors was masked by increased agglutination of the cells by other specificities of antibody present in the sera. We found stroma prepared from papain-treated horse erythrocytes to be devoid of detectable infectious mononucleosis receptor activity. Perhaps conversion of the papain-treated erythrocyte to stroma leads to a rearrangement of antigenic determinants and the loss of accessibility to antibody.

Summary. The effects of three proteases, trypsin, papain, and pronase, upon the major glycoprotein of horse erythrocyte membranes were examined. By three independent criteria, papain was the most effective. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of stroma derived from papain-treated erythrocytes revealed the loss of the major glycoprotein band characteristic of this membrane. Concomitant with the loss of the major glycoprotein band, fragments were released from the erythrocyte which had a carbohydrate composition similar to that of the intact glycoprotein. Also, reactivity of the treated erythrocytes with infectious mononucleosis antibody, a property associated with the major glycoprotein, was reduced. Only a portion of the membrane glycoprotein was susceptible to pronase action and none was cleaved by trypsin.

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