Specific Release of Heterogenetic "Mononucleosis Receptor" by Influenza Viruses, Receptor Destroying Enzyme and Plant Proteases. (23405)

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The usefulness of proteolytic enzymes in the detection of certain blood group antigen-antibody interactions(1) has led several investigators to study the applicability of trypsin and papain to a more reliable demonstration of mononucleosis antibody(2-5). Wöllner reported that papain destroys specifically the receptor for mononucleosis on sheep erythrocytes(4). On this basis, he developed a highly sensitive and specific diagnostic test for mononucleosis(6). Our results confirm the specificity of this test(5). They indicate, however, that enzymes besides papain are active and that at least part of the specific structure of the erythrocyte receptor for mononucleosis is not destroyed by enzymatic action, but released into the supernatant fluid(5).

This paper reports on specific removal of the erythrocyte receptor for mononucleosis by virus receptor destroying enzyme (R.D.E.) from *Vibrio cholerae,* by influenza viruses, and by the plant proteases bromelin, papain and ficin, and describes some serological and qualitative chemical properties of the material released by enzyme action.

Materials. Sera. Freshly drawn and clotted blood was obtained from patients with clinical, serological and hematological signs of mononucleosis. As controls bloods from healthy individuals and those suffering from a variety of diseases were included. Serum was removed by centrifugation, heat inactivated at 56°C for 30 min. and either tested immediately or stored at −15°C. *Erythrocytes*: A pool of citrated blood from 4 sheep (12 sheep used on a rotating basis) was obtained weekly and kept at 2 to 4°C. From this stock a 2% suspension was prepared each day after washing the erythrocytes 3 times with saline. Citrated beef erythrocytes were obtained from a slaughter house. *Solutions*: Saline solution (0.85%) and a m/15 Sørensen phosphate buffer of pH 7.2 were used. The final solutions were prepared as described by Pickles for the detection of incomplete antibodies (1).

Enzymes. The following enzyme preparations were employed: bromelin (Takamine); cathepsin (Delta); papain 1:200 (Fisher Scientific) both without and with glutathione (0.01 m final concentration); erepsin (General Biochemicals); ficin (Mann); ficin crystallized 3x (Worthington); pancreatin (Viscose Powder); rennet (General Biochemicals), chymotrypsinogen, crystallized 6x (Sigma Chemical); chymotrypsin (General Biochemicals); trypsin (Difco 1:250) carboxypeptidase crystallized 3x (Worthington); lipase (Delta Chemical); β-amylase (Bios); emulsin (Delta); hyaluronidase, 2 lots from bull testis (Wyeth); *Streptococcus hemolyticus* hyaluronidase (Wyeth); enzyme preparation from *Lactobacillus bifidus* variatio *pennsylvanicus;* lysozyme crystallized 3x (Worthington); Taka-diastase (Parke Davis); R.D.E. from *Vibrio cholerae* (Behringwerke, Germany) in concentration of about 300 units/ml (plus 0.015 m $CaCl_2$ final concentration). All enzymes were made up according to one of the two methods by Pickles(1) depending on the purity of the preparation.

Influenza viruses. Chick allantoic fluid containing the Melbourne strain of Type A, the Lee strain of Type B and the S_{15} strain of swine influenza virus, respectively (about $1x10^9$ $I.D._{50}$ of virus per ml) was used in different experiments.

Glassware. All solutions were prepared in Pyrex glassware; 0.2 ml serological pipettes and test tubes of 7.5 x 0.9 cm size were used in all serological experiments.

Methods. Enzymatic treatment of erythrocytes: Sheep or beef erythrocytes, 1 to 7 days after bleeding, were washed 3 times, mixed with 5 volumes of the respective enzyme solution and incubated with repeated shaking for 30 min at 37°C. Subsequently, the cells were washed twice with saline. *Treat-*

TABLE I. Specific Removal of Receptor for Infectious Mononucleosis. Sera from cases with infectious mononucleosis (reciprocal titer).

Enzyme	Patient*	Serum unabsorbed — Sheep erythrocytes: Native	Serum unabsorbed — Sheep erythrocytes: Enzyme-treated	Serum absorbed with enzyme-treated sheep erythrocytes: Native	Serum absorbed with enzyme-treated sheep erythrocytes: Enzyme-treated
Receptor destroying enzyme	H.V.	64	16	64	<2
	A.M.	256	16	128	<2
	J.W.	>1024	16–32	512	<4
Influenza viruses:† (Melbourne strain of Type A or S_{10} strain of swine influenza)	W.J.	256	64	128	4
	J.W.	2048	256–512	512	8
	D.K.	128	64	64	<2
Bromelin	J.W.	2048	16	2048	2–4
	G.W.	512	8	128–256	<2
	H.D.	256	16	128	<2
Ficin (crude or 3 times crystallized)	J.H.	>128	16	128	<2
	G.W.	>256	8	64–128	<2
	J.W.	2048	128–256	512–1024	4
Papain (activation with SH groups is unnecessary)	G.W.	256	32	64–128	<2
	J.W.	2048	16	512	2
	C.D.	512–1024	64	2048	4

* Each enzyme was tested on serum of patient J.W. for comparative purposes.

† The Lee strain of Type B virus acted in a similar fashion, though weaker under the same experimental conditions.

ment of erythrocytes with influenza viruses. Packed and washed erythrocytes were incubated at 37°C for 12 to 20 hrs with a 30- to 40-fold volume of chick allantoic fluid containing influenza virus to which streptomycin and penicillin were added. The mixture was occasionally shaken and subsequently washed with saline. *Absorption of serum*: An equal amount of serum and packed sheep erythrocytes treated as described above were mixed well, kept at room temperature (22-25°C) for 1 hour and frequently shaken. After centrifugation, the erythrocytes were discarded and the serum was used for titration. Within each experimental series sheep erythrocytes treated with the same enzyme were used for absorption and titration.

Titration. Each serum was divided into 2 portions: unabsorbed and absorbed. For each of these, 2 geometrical dilution series (two fold) were set up. To each tube 0.2 ml saline was added and subsequently 0.2 ml serum to the first tube in each row. After thorough mixing, 0.2 ml was transferred from the first to the second tube and so forth. The same pipette was used throughout for each row. A volume of 0.2 ml of a 2% suspension of native erythrocytes was added to one of each of the 2 sets of dilutions of unabsorbed and absorbed serum and the same amount of enzyme treated erythrocytes was added to one of each of the other 2 sets of serum dilutions. The tubes were shaken, covered, and left overnight at room temperature. They were read macroscopically against diffuse light after a little shaking. The last tube showing unequivocal agglutination was taken as end point. Titers are expressed in terms of 2-fold geometrical dilution of serum. Final dilutions are obtained by multiplying these values by 2.

Controls. a) In each serological test were included 1) 0.2 ml saline plus 0.2 ml 2% suspension of native sheep erythrocytes; 2) Same as above except that the erythrocytes were treated with the enzyme under investigation; b) In the enzymatic studies, enzyme preparations boiled for 30 minutes and uninoculated allantoic fluid were included.

Hemagglutination inhibition. The inhibition method and controls employed have been described earlier(7), however 0.2 ml of all solutions or suspensions was used instead of 0.1 ml. After addition of inhibitor and also after addition of a 2% suspension of sheep erythrocytes, there was an incubation of about 10 hours at 4°C and room temperature re-

TABLE II. Increase of Agglutinin Titers for Receptors Other Than for Infectious Mononucleosis following Enzymatic Digestion.
Sera from cases without infectious mononucleosis (reciprocal titer).

Enzyme	Patient	Serum unabsorbed, Sheep erythrocytes: Native	Serum unabsorbed, Sheep erythrocytes: Enzyme-treated
Receptor destroying enzyme	O.B.	64	256
	E.B.	4	8
	A.S.	4	8–16
Influenza virus	C.B.	2	8
	A.X.	2	4–8
Bromelin	O.B.	64	256
	E.B.	4	16
	A.S.	4	8–16
Ficin	P.P.	2	64
	E.B.	2	128
	A.S.	4	32
Papain	J.R.	2	16
	P.N.	128	1024
	P.D.	4–8	128–256

spectively. Readings were made with the naked eye. Blood group antisera and human blood group mucoids employed in this study have been described previously(7,8).

Preparation of enzymatic digests from erythrocytes. The supernate of enzymatically treated erythrocytes was boiled in a water bath for 30 min. Insoluble material was removed by centrifugation and the suitably concentrated solute tested as such or after dialysis through cellophane casing (Visking, average pore size 48 Å) for 72 hrs at 4°C against 60 volumes of distilled water (4 times changed). The color reactions employed in this investigation were standard laboratory procedures unless stated otherwise.

Results. Serological: Table I gives some typical serological observations. The enzyme preparations listed appear to remove the receptor for mononucleosis from the erythrocyte surface almost completely. This is evident, if one compares in unabsorbed sera from patients with mononucleosis the titer difference against native and against enzyme treated erythrocytes. Enzyme treated erythrocytes never gave a higher but usually a lower titer than native cells, due to removal of the receptor for the specific mononucleosis antibody. Sheep agglutinins which were not due to mononucleosis generally show an increase in titer against enzyme treated erythrocytes (Table II).

Unequivocal results were obtained uniformly if the sera were absorbed, prior to testing, with erythrocytes treated with one of the active enzyme preparations. Such a procedure removes all demonstrable agglutinins against sheep erythrocytes except those against the mononucleosis receptor. This is shown by the data on the right half of Table I.

None of the other enzyme preparations listed under "Materials" released the mononucleosis receptor or any sheep erythrocyte receptor reacting with agglutinins of human serum under the experimental conditions. All boiled enzyme preparations, boiled virus suspensions and uninoculated allantoic fluid were inactive.

The results of inhibition studies are listed in Table III. It can be seen that enzymatic digests from sheep and beef erythrocytes specifically inhibit the mononucleosis antibody. Beef cell digests inhibit to a larger extent than

TABLE III. Specific Inhibition of Infectious Mononucleosis Antibody.*

	Minimum amt of material giving complete inhibition of hemagglutination (mg/ml)
Sheep erythrocytes	
Papain digest	
non-dialyzable	1
dialyzable	5–10
R.D.E. digest	
non-dialyzable	5
dialyzable	inactive†
Influenza virus digest	
non-dialyzable	2– 5
dialyzable	5–10
Beef erythrocytes	
Papain digest	
non-dialyzable	0.3–1
dialyzable	± 10 mg
R.D.E. digest	
non-dialyzable	5
dialyzable	10
Controls	
Papain (boiled)	inactive
non-dialyzable	
dialyzable	
Influenza virus (boiled)	
non-dialyzable	
dialyzable	
R.D.E. (boiled)	

* 4 minimum hemagglutinating doses.
† Inactive = No inhibition at 10 mg/ml or less.

TABLE IV. Color Reactions Given by Material Released Enzymatically from Erythrocytes.

	Reagents			
	Morgan-Elson without alkali	Bial Neuraminic acid modification(9)	Molisch	Anthrone
Sheep erythrocytes				
Papain digest				
non-dialyzable	+++	++	++(+)	++
dialyzable	+	+	+	+
R.D.E. digest				
non-dialyzable	++	±	+++	+++
dialyzable	++	+	0	0
Influenza digest				
non-dialyzable	++	++	+++	+++
dialyzable	++	+	++	++
Beef erythrocytes				
Papain digest				
non-dialyzable	++	++	+++	++
dialyzable	+	+	+	+
R.D.E. digest				
non-dialyzable	+	+(+)	+++	+++
dialyzable	+	+++	+++	+++
Controls				
Mucoid from meconium	+++	+++	++	++
Papain solution				
non-dialyzable	—	—		
dialyzable	—	—		
Influenza virus				
non-dialyzable	±	—	+	+
dialyzable	+	—	+	+
R. D. E.	±	—	0	0
Allantoic fluid				
non-dialyzable	+	—	+	—
dialyzable	+	—	+	+

0 = Not determined because commercial R.D.E. contains 10% lactose as stabilizer.

those from sheep cells. In both instances, about 90% of the material subjected to digestion by papain and R.D.E. is dialyzable, whereas 90% or more of the inhibitor is not dialyzable. The inhibiting power of these crude preparations is low, but apparently specific. Human serum sickness and Forssman antibodies and blood group antibodies anti A, and B, anti H(O) and anti D(Rh_0) were not inhibited by these digests.

Human blood group mucoids A, B and H(O) from ovarian cysts and meconium and purified blood group substance from horse (starting material was Sharp and Dohme mucoid from horse, lot 346330) in concentrations of 10 mg per ml and below did not inhibit the mononucleosis antibody.

Chemical observations: The erythrocyte digests did not lose their activity by heating in a boiling water bath for 30 min. Besides boiling, dialysis and removal of insoluble matter, no fractionation or purification procedure was carried out. Reactions for carbohydrate which were performed on such solutions of comparable concentrations (about 1% dry weight) are shown in Table IV. They indicate the presence of neuraminic acid or its derivatives in these digests.

The biuret test on the non-dialyzable serologically active material was negative, while the ninhydrin reaction was weakly positive to positive. The active materials released by all enzymes investigated gave similar reactions.

Discussion. The fact that R.D.E. from *Vibrio cholerae* as well as influenza viruses and plant proteases remove specifically the erythrocyte receptor for mononucleosis poses the question as to mode of action of these enzymes and the nature of their substrate. The activity of influenza viruses and R.D.E. point

to neuraminic acid or its derivatives as a structure possibly involved in the specific activity of the receptor, since the only enzyme influenza viruses are known to possess is neuraminidase(10). It is in agreement with this consideration that materials released during enzymatic inactivation give strong color reactions considered to be characteristic for neuraminic acid or its derivatives. Some of these color reactions are weakly positive when applied to normal allantoic fluid or allantoic fluid containing influenza virus. This may be explained either by material released from the allantoic membrane into the fluid or by substances contained in the influenza virus itself. The very weakly positive Morgan-Elson reaction (without alkali) in preparations of R.D.E. may have its origin in cholera vibrio, in the growth medium or in the enzyme stabilizer.

It is remarkable that proteinases from animals did not affect recognizably the mononucleosis receptor from erythrocytes while plant proteinases were active. It is unlikely that these proteinases contain an enzymatically active impurity because 3x crystallized ficin removes the receptor.

An immunological and presumably a chemical relationship may be postulated between the agent causing mononucleosis and the specific receptor on the erythrocyte surface, otherwise occurrence of a specific heterophilic antibody against sheep erythrocytes in sera of patients with mononucleosis would be difficult to explain. There is no indication that sheep erythrocytes are agglutinated by virus circulating in the blood stream of patients with mononucleosis. It is of interest that crude enzyme digests of sheep and beef erythrocytes inhibit the mononucleosis antibody. While the inhibition is not very high, it is nevertheless specific. Activity of these digests varied considerably in different experiments. Optimal conditions remain to be established.

The heat stability of the serologically active substances is in agreement with earlier observations by Schwarzweiss and Tomcsik (11), who obtained material capable of inhibiting mononucleosis antibody by extracting sheep and beef erythrocytes with hot organic solvents. The present experiments have shown in agreement with earlier authors(12) that beef erythrocytes yield more active inhibiting material on a weight basis, than those from sheep. Furthermore, our methods of preparation were entirely different from those of previous investigators.

The mononucleosis inhibitor is probably present predominantly in macromolecular form as it is essentially nondialyzable. Its heat stability and color reactions indicate that carbohydrate may be involved in the specificity of this receptor.

Summary. Release of heterogenetic infectious mononucleosis receptor from sheep and beef erythrocytes by receptor destroying enzyme, influenza viruses and plant proteases has been described. Numerous other enzymes were inactive. Material released by enzymatic action inhibited specifically infectious mononucleosis antibody and gave color reactions indicating that neuraminic acid or its derivatives are involved.

We are indebted to the following: Dr. Werner Henle and Dr. Florence Lief, University of Pennsylvania for advice and for the virus preparations as well as for the allantoic fluid and R. D. E., Dr. J. Seifter, Wyeth Institute for Medical Research, Radnor, Pa., for the hyaluronidases, and to Takamine Laboratories, Clifton, N. J., for a sample of bromelin.

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Received July 8, 1957. P.S.E.B.M., 1957, v96.