159. *Effect of Trypsin on M and N Blood-Group Antigens on Human Erythrocytes*

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Mäkelä *et al.*¹⁾ have reported that crystallized trypsin destroys M and N antigens on human erythrocytes and releases undialyzable sialic acid from the erythrocytes. We²⁾ have previously demonstrated that treatment of human erythrocytes with crystallized trypsin in a low concentration, results in destruction of the I activity of the erythrocytes and release of an I-active sialoglycopeptide from the erythrocytes. Recently, Winzler *et al.*³⁾ have reported that sialoglycopeptides are liberated from human erythrocytes by incubation with crystallized trypsin in a high concentration. However, they have not examined the blood-group activities of the trypsin-treated erythrocytes and of the released sialoglycopeptides. The present study was undertaken to clarify the relationship between the blood-group activity of sialoglycopeptides liberated from human red blood cells by trypsin treatment and the agglutinability of the trypsin-treated red cells against anti-M and -N immune sera.

Experimental methods. Hemagglutination test of human red blood cells before and after trypsin treatment and hemagglutination inhibition test of isolated sialoglycopeptides were performed by using rabbit anti-M and -N immune sera. For analytical and preparative separation of sialoglycopeptides, the following electrophoresis with Whatman No. 3MM paper (30 cm, width $\times$ 46 cm, length) was used. Paper electrophoresis was carried out by the horizontal open strip method with pyridine-acetic acid-water (10:0.4:90, by vol.) buffer⁴⁾ at 300 V for 6 hours and platinum electrodes were used. After electrophoresis the paper was dried at room temperature and either dyed with the staining reagents for the analytical purpose or cut off to small pieces for the isolation of sialoglycopeptides. Ultraviolet absorption spectra of sialoglycopeptides were measured by HITACHI recording spectrophotometer EPS-2.

Results and Discussion. From 100 to 300 ml of washed and packed M, N, or MN human red blood cells was incubated an equal volume of 0.1 M Na_2HPO_4 solution adjusted to pH 7.7, containing 1 mg of trypsin ($2 \times$ crystallized preparation, Sigma Chemical,

Table I. Agglutination of human erythrocytes against anti-M and -N sera before and after trypsin treatment

Anti-M		Anti-N		Anti-M		Anti-N	
No.	Before T.T.	After T.T.	Before T.T.	After T.T.	No.	Before T.T.	After T.T.
1	+3	0	0	+3	23	0	0
2	+3	0	0	+3	24	0	0
3	+2~+3	0	0	+3	25	0	0
4	+2~+3	0	0	+3	26	0	0
5	+3	0	0	+3	27	+3	0
6	+3	0~+1	0	+3	28	+3	0~+1
7	+3	0	0	+2~+3	29	+3	0
8	+3	0	0	+3	30	+3	0~+1
9	+2~+3	0	0	+2	31	+3	0
10	+3	0	0	+3	32	+3	0
11	+3	0	0	+3	33	+3	0
12	+3	0	0	+3	34	+2~+3	0
13	+3	0	0	+3	35	+3	0
14	+3	0	0	+3	36	+3	0
15	+3	0	0	+3	37	+3	0
16	0	0	+3	+2~+1	38	+2~+3	0~+1
17	0	0	+3	+3	39	+2~+3	0
18	0	0	+3	+2~+3	40	+3	0
19	0	0	+3	+2	41	+3	0~+1
20	0	0	+2	+2~+3	42	+2~+3	0~+1
21	0	0	+3	+3	43	+3	0
22	0	0	+3	+3	44	+3	0

Agglutinability symbols: +3, Strong agglutination; +2, middle agglutination; +1, weak agglutination; 0, no agglutination; T.T., trypsin treatment.

Nutritional Biochemical, and Worthington Biochemical Cos.) per 1 ml of the Na_2HPO_4 solution. After incubation for 90 minutes at 37°C under stirring, the mixture was cooled in ice-water and centrifuged. Before and after incubation with trypsin, the red cells were qualitatively tested with rabbit anti-M and -N immune sera. The agglutinabilities of the untreated and treated red cells against anti-M and -N sera are presented in Table I. The M activity of the red cells, in all samples tested, was inactivated by the trypsin treatment and the treated red cells became agglutinable with anti-N serum. The activity of the N red cells was not destroyed under the same conditions of the trypsin treatment, but their agglutinability against anti-N serum was slightly decreased. The N red cells treated with trypsin did not react with anti-M serum. Under the same conditions, the MN red cells were converted into the red cells which reacted only with anti-N serum. Some of these results are in accord with Yokoyama and Trams's findings⁵⁾ that trypsin affects M antigen on human erythrocytes, but not N antigen. However, Mäkelä *et al.*¹⁾ and

Morton⁶⁾ have reported that both M and N antigens are destroyed by trypsin treatment. As suggested by Yokoyama and Trams,⁵⁾ this discrepancy may be due to the use of the different preparations of crystallized trypsin and of the particular samples of anti-sera.

To examine the blood-group activity of sialomuco-substances released from intact red cells by the trypsin treatment, the supernatant solution separated from the incubation mixture by centrifugation was submitted to the following isolation procedure. The red-tinted supernatant was brought to 5% trichloroacetic acid (TCA) solution by adding solid TCA in ice-water and filtrated. The cooled colorless filtrate was three times shaken with ether to remove TCA, neutralized to pH 5.5–6.0 with 2N-NaOH, concentrated to about 50–60 ml at 30°C in a rotary evaporator, and dialyzed against 5 liter of distilled water at 3°–5°C for 24 hours. The dialysis was three times repeated. The dialyzed solution was concentrated at 30°C in a rotary evaporator and dried over P₂O₅ in a vacuum desiccator. The dry residue which will be referred to as the crude preparation, was subjected to paper electrophoresis to test its components. After electrophoresis, the dry paper was divided lengthwise into 3 ribbons which separately stained with the methylene blue,⁴⁾ aminoblack-10B, and ninhydrin reagents. The three crude preparations obtained from M, N, and MN red cells gave all the same electropherograms (Fig. 1). These preparations were each separated into 7 components, i.e., the spots 1, 2, 3, 4, 5, 6, and 7. In these crude preparations, the spots 1, 2, and 3 migrated toward the anode, while the spots 4, 5, 6, and 7 migrated toward the

Fig. 1. Paper electropherograms of three crude preparations obtained from M, N, and MN red blood cells by trypsin treatment (see text).

cathode. The spot 1 of the three crude preparations each gave a positive reaction with the methylene blue reagent suggesting the presence of sialomuco-substances⁴⁾ and slightly reacted with the aminoblack-10B and ninhydrin reagents indicating a content of peptide or protein. The other spots except the spot 1 stained with the aminoblack-10B and ninhydrin reagents, but not with the methylene blue. These results indicate that the spot 1 which has a net negative charge, is likely to be a sialoglycopeptide and the other spots appear to be peptides. The component 1, i.e., the spot 1 was isolated by preparative paper electrophoresis. An aqueous solution containing 30 mg of the crude preparation from M, N, or MN red cells was applied as a band on one paper strip. After electrophoresis, three small guide strips cut from the both ends and center of the strip, were stained by the methylene blue⁴⁾ and the undyed zone with mobility corresponding to the spot 1 was cut off to small paper pieces which were extracted three times with distilled water at 3°–5°C under stirring. The extracts were combined, filtered, concentrated in a rotary evaporator at 30°C, and dried over P₂O₅ in a vacuum desiccator. Forty five mg of the dry residue was dissolved in 50 ml of distilled water. The solution was passed through a Dowex-50W X-8 resin column (100~200 mesh, 2 cm, diameter × 5 cm, height, hydrogen form) and the column was then washed with distilled water. Chromatography was carried out at 3°–5°C. The eluate and washings were combined, adjusted to pH 6.0 with 2N·NaOH, concentrated to 50 ml in a rotary evaporator at 30°C, and dialyzed against 5 liter of distilled water at 3°–5°C for 24 hours. The dialyzed solution was concentrated in a rotary evaporator at 30°C and dried over P₂O₅ in a vacuum desiccator. The dry residue with light straw color will be referred to as the purified preparation. The three purified preparations obtained from M, N, and MN red cells were submitted to paper electrophoresis to examine these purity. These

Fig. 2. Paper electropherograms of three purified preparations obtained from M, N, and MN red blood cells (see text).

preparations each gave one single spot with all the same electrophoretic mobility and also exhibited all the same reactivity against the three staining reagents (Fig. 2). The crude preparations gave all the same ultraviolet absorption spectra which showed λ max 277 $m\mu$. The three purified preparations showed identical ultraviolet absorption spectra which had a weak plateau-like absorption between 256 $m\mu$ ~280 $m\mu$. The results of paper electrophoresis and absorption spectra indicate that the combination method of preparative paper electrophoresis and Dowex-50W column chromatography is a useful tool for the group separation of sialoglycopeptides and that the three purified preparations are a homogeneous sialoglycopeptide. Yields of the purified sialoglycopeptides from each 100 ml of packed M, N, and MN red cells were 13.9 mg, 13.5 mg, and 14.2 mg, respectively.

The inhibitory activity of the three purified sialoglycopeptides against rabbit anti-M and -N immune sera was examined and the results are given in Table II. The sialoglycopeptide from M red cells showed only a M blood-group activity. The sialoglycopeptide from N red cells exhibited a N activity, but not M activity. The sialoglycopeptide from MN red cells exhibited both M and N activities.

Table II. Hemagglutination inhibition test 1* of purified sialoglycopeptides (SGP) obtained from M, N, and MN human red blood cells

Anti-serum	SGP from M cells	SGP from N cells	SGP from MN cells
Anti-M* ²	0.63~1.25	15<	1.25
Anti-N* ²	15<	1.25	1.25

*1 mg/ml of purified SGP inhibiting 4 agglutinating units.

*2 Ortho Pharmaceutical Co. (Rabbit anti-immune serum).

It was clarified from above experiments that the undialyzable M-active sialoglycopeptide (SGP) was released from M human red blood cells (RBC) by the treatment with crystallized trypsin and that the trypsin-treated red cells abolished the M activity and became agglutinable with anti-N serum. Under the same conditions of the trypsin treatment, a relatively large amount of the undialyzable N-active sialoglycopeptide was liberated from N red cells, although the N activity of the N red cells was hardly affected. The MN red cells were converted into the N-active red cells by the trypsin treatment and the undialyzable MN-active sialoglycopeptide was simultaneously released from the red cells. These experimental results are summarized as follows:

Baranowsky and Lisowska⁷⁾ have demonstrated that M-active sialoglycoprotein obtained from human erythrocyte stroma by the phenol extraction method is cleft to M- and N-active sialomucoid components and under the same conditions N-active sialoglycoprotein is split into two sialomucoids with a low N activity. From these results, they have suggested that the N structure in the M substance may be masked by terminal components of the molecule chains and that the N substance is a precursor substance of MN system. The results of this work and also the previous experiments with pronase⁸⁾ are in good agreement with the above Baranowsky and Lisowska's experiments⁷⁾ and support their suggestion.⁷⁾ On the basis of these observations, it is again possible to build up a hypothesis that the N-like antigen which newly appeared by the treatment with trypsin or pronase,⁸⁾ is a precursor substance of M·N blood-group antigens of human erythrocytes and the N-like antigen is covered with the specific M- or N-active sialoglycopeptide to form intact M or N erythrocyte antigen.

Summary. Crystallized trypsin inactivates M blood-group activity of human red blood cells and renders the treated red cells agglutinable with anti-N serum, and, on the other hand, releases a undialyzable M-active sialoglycopeptide from the red cells. The blood-group activity of N red cells against anti-N serum is hardly affected by the trypsin treatment, but a passable amount of a undialyzable N-active sialoglycopeptide is simultaneously liberated from the red cells.

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