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Action of Enzymes from *Clostridium tertium* A on the Group A Antigenic Determinant of Human Erythrocytes

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ABSTRACT. It is well known that *Clostridium tertium* A produces A-decomposing enzymes. We investigated the changes in ABO-antigenicity of human erythrocytes caused by the culture filtrate from this bacteria. Group A erythrocytes were specifically affected, and a decrease in A-activity and an enhancement of H-activity were observed. No change was observed on group B and O cells. There was a significant difference between the agglutinabilities of group A_1 and A_2 erythrocytes treated with *Cl. tertium* culture filtrate. A_2 erythrocytes completely lost their agglutinability against undiluted anti-A sera. In contrast, A_1 erythrocytes preserved a low A-antigenicity. It was clear that this difference reflected the heterogenous nature of A-antigenicity on group A cell membranes. On the other hand, it was suggested that the A-decomposing enzymes in the culture filtrate also had a heterogenous nature. Our crude enzyme preparation was completely inhibited by D-galactosamine, and weakly by N-acetyl-D-galactosamine or D-galactose. Further enzyme purification was required to elucidate the differences in the antigenic determinants or the structural basis between A_1 and A_2 erythrocytes.

Key words : ABO blood groups — A_1 and A_2 erythrocytes — A-antigenicity — A-decomposing enzyme

In the case of samples from putrefied blood and muscles, it is well known that additions to or subtractions from blood group activities caused by a bacterial action often occur.[1–3] The hazard of modification of blood group activity is undoubtedly greatest when the victim is in an advanced state of putrefaction. In addition, the effect of bacteria on blood grouping techniques sometimes results in misjudgment.[4]

On the other hand, the use of enzymes that alter the serologic properties of blood group antigens has been valuable in the structural elucidation of antigenic substances. Iseki *et al.*[5] isolated a soil bacterium identified as *Clostridium tertium* which produced an A-decomposing enzyme for the A activity of human saliva and of blood group substances extracted from human erythrocytes or hog gastric mucin. Iseki and Masaki[6] found that the disappearance of the A activity was accompanied by an increase in H activity as measured by a hemagglutination inhibition test. Group A erythrocytes are divided into the major subgroups A_1 and A_2. It is reasonable to presume that the difference between the two groups depends not only on the quantity of A antigenic substances on cell membranes

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but also on the quality of A antigens.[7–10] N-acetyl-D-galactosaminyltransferase catalyzes the transfer of N-acetyl-D-galactosamine to exogenous acceptors and may be responsible for blood group A antigenicity. Differences in the enzyme between the two groups have been reported in the point of activity, the optimal pH, the iso-electric point and the Km value.[11,12] Kabat *et al.*[13] and Yoshida[14] described the differences in the structural basis of the A_1 and A_2 antigens.

We improved on the medium conditions so that greater A-decomposing enzyme activity was obtained in the culture filtrate (CF).[15] The present study describes the destruction of group A activity by the CF prepared under our laboratory medium conditions, with special reference to the differences between A_1 and A_2 erythrocytes

MATERIALS AND METHODS

Cultivation of bacteria: The strain of *Cl. tertium* A isolated originally by Iseki was obtained from Dr. Ken Furukawa. The organisms were cultivated in the medium described previously.[15] Culture medium inoculated with the bacteria was incubated anaerobically for 4 to 6 days. The bacteria were sedimented by centrifugation at 3000 rpm for 30 min and the supernatant was passed through a milipore filter ($\phi = 0.45\mu$). The supernatant after dialysis against phosphate buffered saline (PBS, pH 7.4) at 4°C, was used as the A-decomposing enzyme solution, except when we studied the effect of dialysis.

Human erythrocytes: Blood of the appropriate groups, except for the A_2 type, was obtained from normal persons. The A_2 type cells used were commercial products of Ortho Diagnostics, USA. Erythrocytes were washed three times in PBS to remove their serum and buffy coat.

Antisera: Anti-A and anti-B sera of human origin were commercially obtained from Dade, USA and anti-A rabbit immune sera were purchased from Tokyo Hyojun Kessei, Japan. Anti-H lectin was an extract of *Ulex europaeous* seeds prepared in our laboratory. Anti-A_1 lectin from *Dolichos biflorus* seeds was prepared by soaking 10 g of finely ground seeds in 50 ml of PBS overnight at 4°C under gentle agitation and then centrifuging them at 1000 rpm for 10 min. Anti-A_1 lectin was obtained by 30–80% saturation of a supernatant with ammonium sulfate followed by dialysis against PBS. The lectins were kept frozen at −20°C until used.

Treatment of erythrocytes: Human erythrocytes were suspended to a final concentration of 2 per cent in the diluted CF using PBS and were incubated overnight at 4°C. As controls, the same donors' cells were suspended in PBS and incubated under similar conditions. At the end of the incubation period, both CF treated and non-treated cells were washed with PBS and then tested for agglutinability by antisera as follows: serial doubling dilutions of anti-A, -B sera and anti-H, -A_1 lectin were made in a conventional manner. One volume of diluted sera or lectin was added to 1 volume of a 2 per cent suspension of erythrocytes and the mixture was left for 1 hour at room temperature. Then the agglutination titer was determined by the naked eye.

Detection of antibody in eluate: Stains were made by allowing 30 μl of red cell pellets to soak into cotton cloth, after which they were dried for a minimum of 2 days before use. The stain was incubated with 7 drops of anti-A

sera for 2 hours at 37°C, and then for 2 hours at room temperature. After washing the stain with cold PBS, 300 μl of PBS was added and the test-tube was put in a waterbath for 30 min at 56°C. A semi-quantitative analysis of antibody in eluate was made by the method described previously.[4]

Gold-labelling of erythrocytes: Untreated and CF treated group A human erythrocytes were extensively washed with Tris-saline (pH 7.3) and fixed in 2 per cent glutaraldehyde for 2 hours. After washing with Tris-saline, the fixed erythrocyte suspensions were incubated with 1/15 diluted anti-A rabbit immune sera for 4 hours at room temperature under slight agitation, followed by enough rinsing steps. Afterward a large droplet of the gold (Au_5) conjugated anti-rabbit IgG sera (Janssen Life Science Products, Belgium) was applied to the erythrocytes and the mixture was incubated overnight at room temperature, followed by enough rinsing to remove the unbound rabbit IgG-gold complex. Group O human erythrocytes were treated in the same manner. Gold conjugated anti-rabbit IgG sera were used at 1/5 dilution.

Preparation of specimens for TEM: Erythrocytes labelled with Au_5 were dehydrated by increasing the concentration of ethanol, after which they were embedded in Epon. Ultrathin sections were cut on a Porter-Blum microtome and mounted on copper grids. They were poststained with uranyl acetate in a 10% methanol and lead citrate solution. All specimens were examined under a transmission electron microscope (Hitachi H-500, Japan).

RESULTS

ABH(O) agglutinability of CF treated human erythrocytes

Incubation of a suspension of human A_1 erythrocytes with CF from *Cl. tertium* A led to a gradual decrease in their anti-A agglutination titer (Fig. 1). The time required for the disappearance of A activity varied proportionally with the CF concentration examined. However, agglutination of A_1 erythrocytes with anti-A sera did not disappear completely even after incubations for 18, 48 or 72 hours at 4°C. A feeble or clear agglutination was always observed after mixing with anti-A sera diluted from 1 to 8 times.

Table 1 summarizes the results obtained on human erythrocytes treated with CF overnight at 4°C. Treatment of the group A erythrocytes with CF decreased their agglutinability by anti-A sera and consistently increased their reactivity with the anti-H(O) reagents employed. On the contrary, neither disappearance of agglutinability nor acquirement of polyagglutinability with antisera were observed on the CF treated group B and O cells. With respect to group A cells, there was regularly a significant difference between the agglutinabilities of the A_1 and A_2 erythrocytes treated with CF. CF treated A_2 erythrocytes were completely inactive in the agglutination reaction caused by undiluted anti-A sera. However, anti-A sera showed a low titer of agglutinins with CF treated A_1 cells. In order to determine whether the low titer of agglutinins regularly found in incubation with CF treated A_1 cells might be due to residual A antigen, their agglutinability was then measured by titration with anti-A sera absorbed with CF treated group O erythrocytes. As a result, the CF treated A_1 cells were found to have not been rendered wholly inagglutinable in undiluted absorbed anti-A sera. In addition, it was found that anti-A

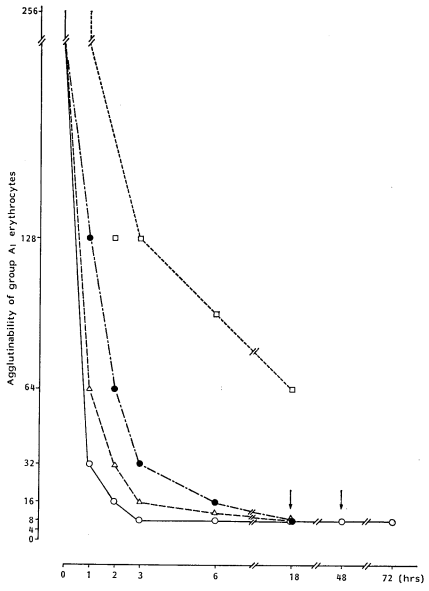


Fig. 1. Time-lapse changes in A-activity of A_1 erythrocytes by culture filtrate. Culture filtrate was used at neat (○), 2^{-2} dilution (△), 2^{-4} dilution (●) and 2^{-6} dilution (□). The arrow indicates an exchange of culture filtrate.

antibody was absorbed and eluted from the stains of CF treated A_1 erythrocytes by the absorption-elution technique (Table 2). These findings gave further support to the view that A_1 erythrocytes distinct from A_2 cells preserve a low A-antigenicity after being treated with CF from *Cl. tertium* A and show a weak agglutination reaction with anti-A sera. When A_1 erythrocytes were incubated with undialyzed CF under dialyzing against PBS overnight at 4°C, the A-antigenicity disappeared completely at a 1.5 or 2.5 fold CF concentration. Without dialyzing against PBS, acquirement of agglutinability was remarkable (Fig. 2). Though we examined the effect of metal ions such as Mn^{++}, Co^{++}, Mg^{++} on the disappearance of the A-antigenicity of A_1 erythrocytes by incubation with dialyzed CF, complete disappearance was not obtained. 2-mercaptoethanol and surfactants had no effect on the enzyme activity.

As shown in Table 1, CF treated A_1 erythrocytes apparently did not react with anti-A_1 lectin. This disappearance of the agglutination reaction was obtained

TABLE 1. Changes in agglutinability of human erythrocytes by culture filtrate

Red cell type	Agglutinin titers							
	Anti-A		Anti-B		Anti-H		Anti-A_1	
	Before	After	Before	After	Before	After	Before	After
A_1 cell	256	8	0	0	16	64	256	0
A_2 cell	128	0	0	0	16	64	0	0
B cell	0	0	256	256	16	16	0	0
O cell	0	0	0	0	64	64	0	0

Culture filtrate was used at 2^{-4} dilution and the mixture was incubated overnight at 4°C.

TABLE 2. Changes in anti-A antibody eluted by the absorption-elution technique

Blood type	twofold serial dilution of the eluate with PBS (2^{-n})									
	n=0	1	2	3	4	5	6	7	8	9
Control	—	—	—	—	—	—	—	—	—	—
A_1	##	##	##	#	+	+	+	+	—	—
B	—	—	—	—	—	—	—	—	—	—
A_1*	#	+	+	+	—	—	—	—	—	—

A_1* indicates type A_1 erythrocytes treated with culture filtrate overnight at 4°C.

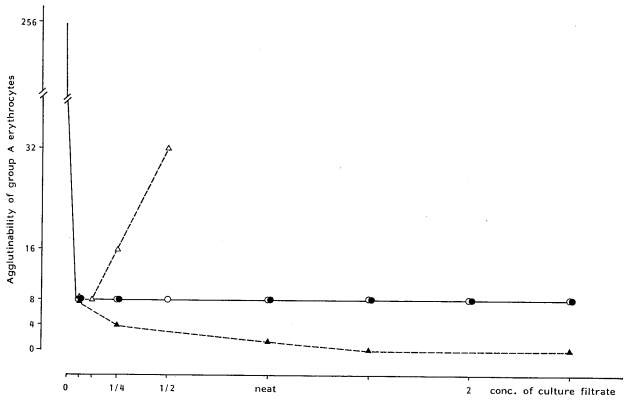


Fig. 2. Changes in A-activity of group A_1 erythrocytes after incubation with culture filtrate overnight at 4°C. Dialyzed (○, ●) and undialyzed (△, ▲) culture filtrates were used. Each of the solid and open symbols, respectively, indicates an incubation with or without dialysis against PBS.

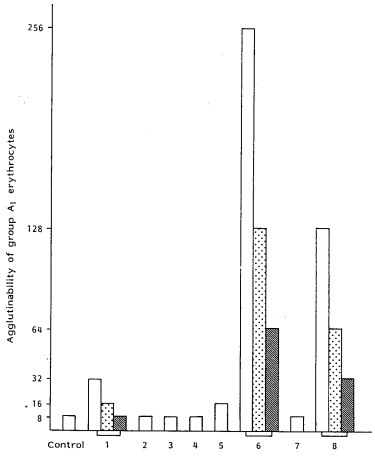
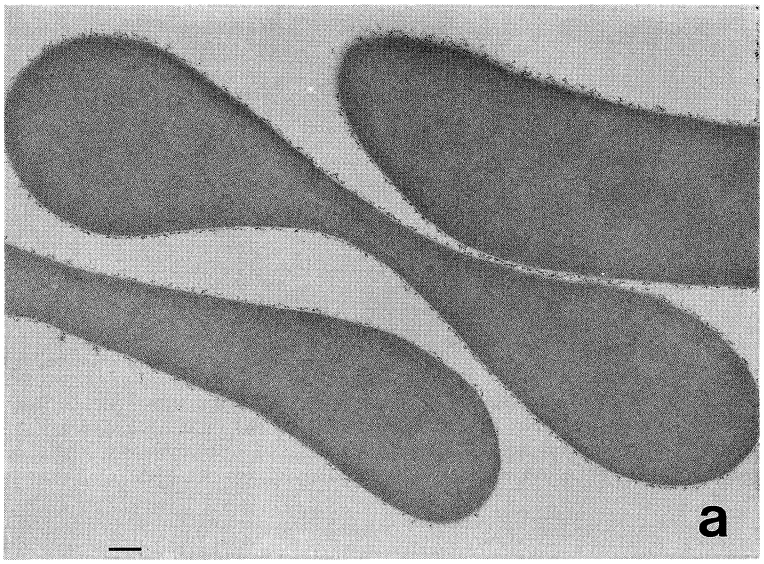


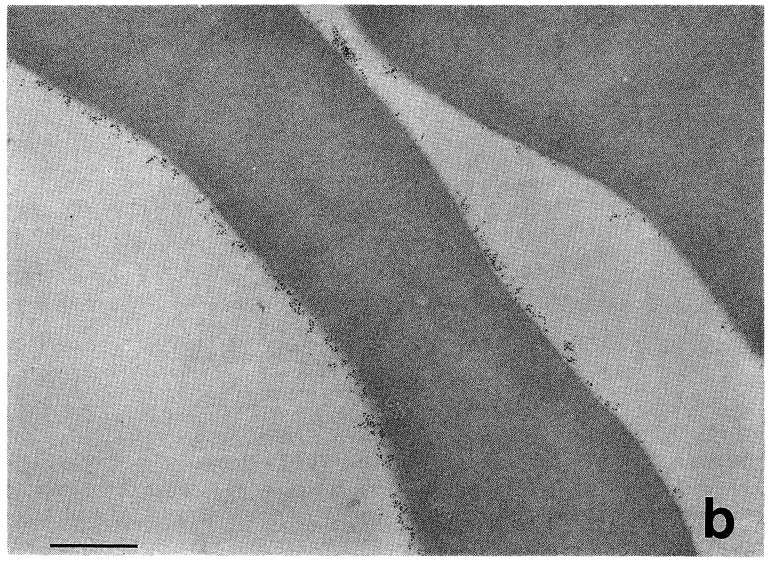
Fig. 3. Inhibition effect of sugars on the A-decomposing enzyme activity. (1) D-galactose, (2) D-mannose, (3) D-fucose, (4) D-glucose, (5) D-glucosamine, (6) D-galactosamine, (7) N-acetyl-D-glucosamine, and (8) N-acetyl-D-galactosamine are examined at 1/36 M (□), 1/72 M (⊡) and 1/144 M (▨) concentration.

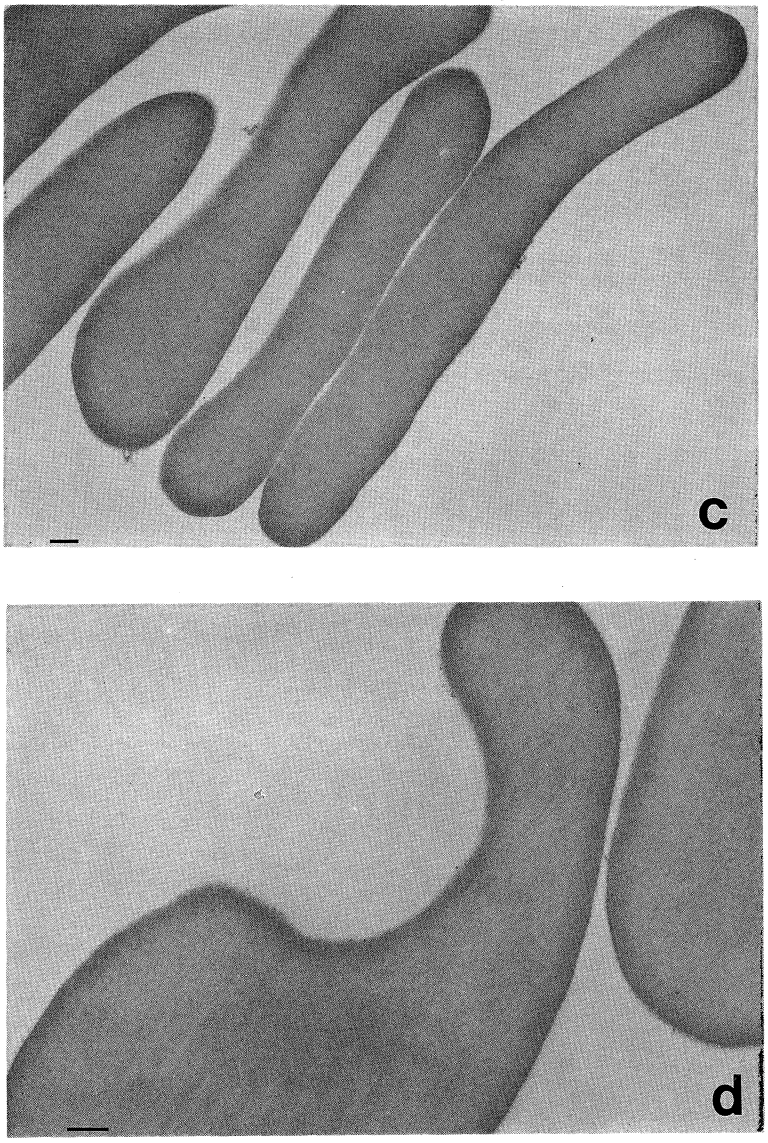
more rapidly than that with anti-A serum and was observed after 1 or 2 hours' incubation with 16 or 32 fold diluted CF.

Inhibition of the enzyme activity by simple sugars

When erythrocytes were treated with CF in the presence of various sugars, only D-galactose, D-galactosamine and N-acetyl-D-galactosamine inhibited the activity remarkably and the inhibition effect was observed in proportion to doses of the sugars (Fig. 3). D-galactosamine showed the strongest inhibition effect among the sugars examined and the enzyme activity was completely inhibited with 1/36M of the sugar. No or little inhibition was observed with D-mannose, D-fucose, D-glucose, D-glucosamine and N-acetyl-D-glucosamine at a 1/36M concentration.

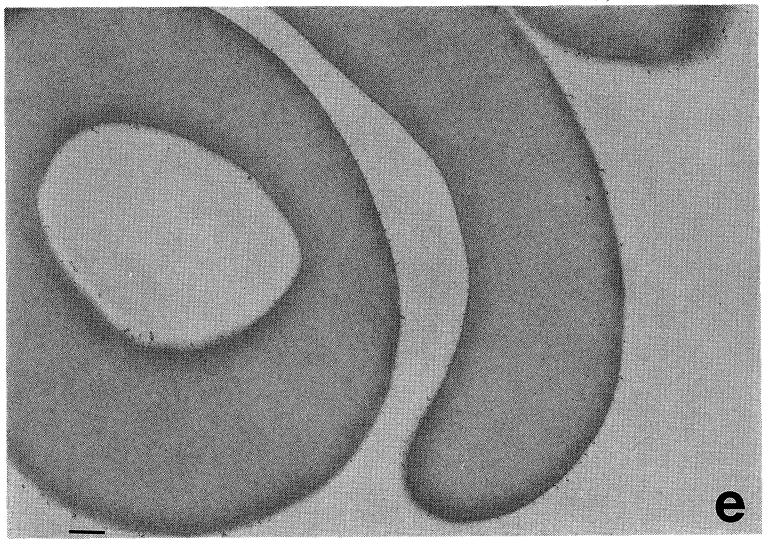






Labelling of human erythrocytes

After application of the anti-A immune antibody, untreated human erythrocytes of group A_1 and A_2 were densely stained by the anti-rabbit IgG serum-gold complex. The cell surfaces were covered with single and clustered gold particles (Fig. 4-a, b). There was no apparent difference between A_1 and A_2 erythrocytes. When human erythrocytes of group O and B were incubated in the same manner, marking was completely absent (Fig. 4-c). After treatment of A_1 and A_2 erythrocytes with CF, however, few gold particles appeared on the cell surfaces (Fig. 4-d, e, f). As shown in Fig. 4-d, e, on the surfaces of A_1 cells, the extent of the loss of the A-antigen sites was in proportion to the concentration of CF used.



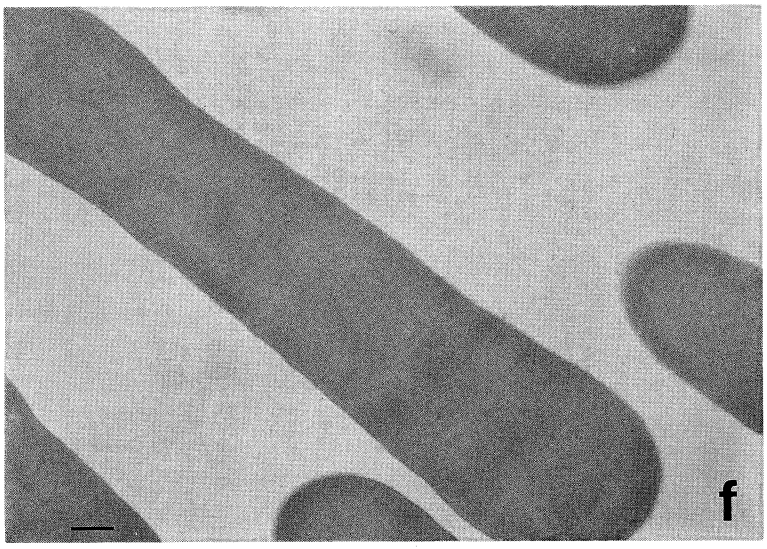


Fig. 4. Thin sections of human erythrocytes marked for blood group A antigen with Au_5 granules. (a) A_1 erythrocytes, (b) A_2 erythrocytes, (c) O erythrocytes, (d) A_1 erythrocytes treated with 2^{-3} diluted CF, (e) A_1 erythrocytes treated with 2^{-4} diluted CF, (f) A_2 erythrocytes treated with 2^{-4} diluted CF. Bars represent 0.3 μm.

DISCUSSION

Since purification of the A-decomposing enzyme still had not been achieved, we used the CF to study the change in A-antigenicity of group A erythrocytes. As for the ABO blood groups, enzymes that specifically spoiled A activity occurred in CF from *Cl. tertium* A. A decrease in the A-activity and an enhancement of the H-activity of group A erythrocytes were remarkable (Table

1). As shown in Table 1, a low titer of agglutinins was observed in CF treated A_1 erythrocytes but not in CF treated A_2 cells. Though our data serves as a very crude estimate of the heterogenous nature of the antigens, the weak agglutinability of CF treated A_1 erythrocytes against anti-A serum illustrates the antigenic difference between group A_1 and A_2 cells. The reason why the residual A antigen is found only on CF treated A_1 erythrocytes is obscure at this time. Available evidence indicates that the A-decomposing enzyme shows an extremely limited substrate specificity.[16] Recently, Clausen *et al.*[17] found a globo-A structure to be present exclusively in A_1 but not in A_2 erythrocytes. More recently,[18] they reported a type 3 chain A structure that could be more important for the structure of A_1 specificity than the globo-A structure. They ascribed the essential qualitative difference between A_1 and A_2 status to the ability of A_1 transferase, and the inability of A_2 transferase, to convert type 3 chain H to type 3 chain A. Our results may reflect a failure to achieve proper conditions for the enzyme to act on small portions of A_1 erythrocytes. The lectin of *Dolichos biflorus* seeds is one of the most studied anti-A_1 specific lectins and A_1 erythrocytes are readily distinguished from A_2 cells by agglutinability with this lectin. The agglutinability, however, disappeared after 1 or 2 hours of incubation with CF. These results support the view that serologically the receptors for *Dolichos biflorus* on human erythrocyte membranes are different from the A-antigen for anti-A serum.[19] It may be that the A-antigenicity which reacts with anti-A_1 lectin exists under better conditions for the enzyme to act.

When A_1 erythrocytes were incubated with undialyzed CF under dialyzing overnight at 4°C, all the A-antigenicity on A_1 cell membranes disappeared (Fig. 2). Incubation without dialyzing resulted in the acquirement of agglutinability. In earlier studies, the enzyme from *Cl. tertium* was specifically activated by Mn^{++}, Co^{++} or mercaptoethanol and inhibited by EDTA.[16] As indicated in Fig. 2, it may be that small molecular components actually take part in complete disappearance of the antigenicity.

Many authors have written about the A-decomposing enzymes and it is generally accepted that these enzymes are comprised of two different species.[16,20–24] One is an N-deacetylase that removes the acetyl group from N-acetyl-D-galactosamine without an increase in H activity. It is suggested that the acquired B may have arisen as a result of the action of this enzyme, transforming A-reactive N-acetyl-D-galactosamine into D-galactosamine.[25,26] The other is a galactosaminidase or an N-acetylgalactosaminidase that spoils A active sugar with an enhancement of H activity. It is suspected that this apparent discrepancy is due to different medium conditions or a mutation occurring in the strain of *Cl. tertium*. Although the enzyme used in our experiment was obscure, as shown in Table 1, there was high activity of a galactosaminidase or an N-acetylgalactosaminidase in our CF regardless of whether an N-deacetylase was existent or not.

In addition to the A-decomposing enzymes, other partially purified enzyme activities have been found in CF from *Cl. tertium* during the course of purification of enzymes which act on blood group substances. Marcus *et al.*[27] reported a method for partial purification of beta galactosidase and beta glucosaminidase. Treatment of erythrocytes with preparations containing both enzymes decreased their ability to react with anti-I cold agglutinins. Howe *et al.*[28] found that CF of the Iseki strain of *Cl. tertium* inactivated the A, M, N antigens of

intact human erythrocytes but had no effect on B, O(H), Rh, Kell or Duffy antigens. We obtained similar results : changes in agglutinability of intact human erythrocytes against anti-A, M, N, P or Le^b antibodies and no effect on B, O(H) or Rh antigens (data not shown). Thus, it was evident that a complete or a partial purification of the A-decomposing enzymes was necessary for a more detailed elucidation of the change in A-antigenicity. However, as described previously,[15] the fact that the reactions with human erythrocytes proceeded enough at 4°C and that the changes in erythrocytes themselves were slight, as shown in Fig. 4, indicates that investigations using crude CF from *Cl. tertium* A are well worth doing. For example, neuraminidase activity obviously existed in crude CF, and the disappearance of the M and N antigens of intact human erythrocytes was observed at 37°C, but not at 4°C.

Yamamoto,[23] using a DEAE-Sephadex column, prepared two enzyme fractions that had A-decomposing activity. Both the crude preparation and these two enzyme fractions were inhibited by D-galactosamine and N-acetyl-D-galactosamine, though not completely as shown in our results (Fig. 3). It is also known that an enzyme from *Trichomonas foetus* destroys the serological activity of A substance[29] and it is tentatively characterized as an N-acetylgalactosaminidase.[30] The enzyme activity was reported to be strongly inhibited with N-acetyl-D-galactosamine, weakly with D-galactose, and not at all with a large number of other sugars.[31] Our crude enzyme preparation from *Cl. tertium* A was completely inhibited with D-galactosamine and weakly inhibited with N-acetyl-D-galactosamine or D-galactose. These differences may reflect the heterogenous nature of the A-decomposing enzymes.

The same results observed on human A_1 erythrocytes were obtained on group A erythrocytes of chimpanzees. Erythrocytes of other animals examined such as dogs, cats, rats, rabbits and hogs showed no change in their agglutinability with anti-A and -B sera after incubation with CF overnight at 4°C (data not shown). These results are of interest in the evolution of group A antigenicity. Although elucidation of the difference in the antigenic determinants and the basis of antigenic specificity between A_1 and A_2 erythrocytes is of value, further enzyme purification and characterization is required to answer several remaining questions. Further studies into this problem are necessary.

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