Shin-ichi Kubo,[1] *M.D.*

Changes in the Specificity of Blood Groups Induced by Enzymes from Soil Fungi

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ABSTRACT: Three strains of *Cunninghamella elegance (C. elegance), Penicillium simplicissimum (P. simpl.),* and *Aspergillus niger (A. niger)* were isolated from soil that produced the enzymes acting on blood groups A and B red cells. Culture filtrates from these fungi contained α-*N*-acetyl-*D*-galactosaminidase as an A-decomposing enzyme, which resulted in an almost complete loss of A specificity and an enhancement of blood group H activity as measured by elution tests using monoclonal antibodies. They also contained an α-*D*-galactosidase and an α-*L*-fucosidase, which partially destroyed the blood group B specific activity, but did not influence the blood group H specific activity.

KEYWORDS: pathology and biology, blood, fungi, soils, blood group substance, monoclonal antibody

Blood group substances of the ABO system are known to be present as glycoproteins in secretions and as glycolipids on red cells. Antigenic determinant sugars of blood group substance properties have been determined by using many kinds of glycosidases [*1-5*].

For many years the determination of blood groups in materials buried in the soil bacteria has been very difficult. In such cases, specific enzymes from soil bacteria might well play important roles, since they decompose the serologically active sugars of blood groups [*6-17*]. Still, we have as yet very little information as to what is involved in the effect of soil fungi on human blood group substances. Thus the present study describes the action of α-*N*-acetyl-*D*-galactosaminidase, α-*D*-galactosidase, and α-*L*-fucosidase, isolated from three strains of soil fungi, on the A, B, and H blood group substances of human red cells.

Materials and Methods

Isolation of Soil Fungi

Soil samples were collected to a depth of 15 cm using a core sampler. The soil samples were dried and filtrated through a No. 10-mesh (2-mm) sieve.

For isolation of fungi from the sample the dilution plate [*18*] and alcohol-heat treatment methods [*19*] were both applied.

Samples were extracted with sterile water and dilutions were cultured on malt extract agar (MEA) medium [*20*, p. 38] for 14 days at 25°C.

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[1]Graduate student of medicine, Nagasaki University School of Medicine, Nagasaki, Japan.

In addition, soil samples were washed with 70% ethanol (8 min) and heat treated (60°C, 30 min) before the aqueous extraction; dilutions of these extracts were cultured on potato dextrose agar (PDA) medium [*21*] for 14 days at 25°C.

Both MEA and PDA media contained chloramphenicol (100 μg/mL) to inhibit bacterial growth.

Fungal isolates were maintained by three monthly transfers on PDA slants and stored at 4°C.

Identification of Isolates

Plate Culture—Four kinds of solutions were used for the identification of the plate medium as follows: an MEA medium, a PDA medium, an oatmeal-agar (OA) medium [*21*], and a potato-carrot-agar (PCA) medium [*21*]. Isolates were cultured on the plates at 25°C for 10 or 14 days. The characteristics of these isolates were then examined, and isolates were identified to their species level by referring to monographs and papers [*20,22-26*].

Slide Culture Method—Further microscopic characteristics of the conidia were examined by the slide culture method [*21*].

After incubation at 25°C, the slide culture was examined for identification at an appropriate time.

Cultivation of Isolates on Groups A, B, and O(H) Red Blood Cells (RBC)—Isolates were cultured on Groups A, B, and O(H) RBC following the slide culture method. A filter paper (qualitative 2, Toyo Roshi Co., Ltd), 20- by 20-mm size, was placed on a microscopic slide supported in a moist chamber, and 500 μL of a 50% A-, B-, and H-RBC saline solution was pipetted on a filter paper.

Fungi were inoculated on the RBC that had been soaked in the filter paper and then were incubated at 25°C for 14 days.

The uninoculated groups A, B, and O(H) RBC were used as controls after incubation at 25°C for 14 days.

Determination of ABH Types from the Fungi-Treated RBC—After cultivation of isolates on the RBC for 14 days, the ABH blood group was determined by elution tests from the RBC of the culture medium.

The filter paper of the culture medium, 10 by 20 mm in size, was soaked in a tube containing 5 mL of sterile pure water. After standing overnight at 4°C, 500 μL of the supernatant exudation was transferred to a piece of gauze (Type 1, J. P. XI). The gauze was dried overnight at room temperature sheltered from direct sunlight. Three threads, each of 5-mm length, from the gauze were used for the elution tests as antigen.

The mouse monoclonal antibodies, supplied by Professor Shigenori Ikemoto of the Laboratory of Human Biology, Jichi Medical School, were immunoglobulin M (IgM) antibodies and used for the elution tests. The titers of anti-A, B, and H monoclonal antibodies were ×128, ×56 − ×128, and ×128, respectively. The monoclonal antibodies had higher specificity than those of human origin [*27*].

The elution tests were performed following Yada's method [*28*]. Three threads, each of 5-mm length, were placed in a test tube and one drop of antiserum was added. Following incubation at 4°C overnight, these specimens were cautiously rinsed with a cold physiologic saline solution five times to ensure complete removal of the unabsorbed antibody. After rinsing, one drop of the saline solution was added and the deposits mixture was incubated in the water bath at 55°C for 10 min. After incubation, one drop of 0.5% suspension of groups A, B, and H cells was added to each tube, and then the tube was centrifuged at 1000 rpm for 1 min. By holding the tube gently over a microscopic concave mirror, the presence or absence of agglutination was ascertained.

Qualitative Analysis of Glycosidase from the Isolates—Qualitative analysis of glycosidase, which decomposes ABH blood group substances from isolates, was attempted following an

o-nitrophenyl-β-*D*-galactopyranoside (ONPG) test that was used for the detection of β-galactosidase from bacteria.

For assays of enzymic activity, 25μ*M* of *p*-nitrophenyl (PNP)-α-*N*-acetyl-*D*-galactosaminide, 50μ*M* of PNP-α-*D*-galactopyranoside, 50μ*M* of PNP-α-*L*-fucopyranoside, and 50μ*M* of PNP-β-*N*-acetyl-*D*-glucosaminide were used as the substrate. A mixture of 2.5 mL of substrate solution and 7.5 mL of peptone solution (pH 6.75) was used for the liquid culture medium. Isolates were inoculated into this liquid culture and then incubated at 25°C at a 165-rpm shaking rate for seven days. After incubation, the culture filtrates were collected through a membrane filter (0.45 μm).

Isolates were also cultured in peptone solution without substrates under the same conditions. The liquid culture medium without inoculation was incubated at 25°C at a 165-rpm shaking rate for seven days. After incubation, culture filtrates from peptone solution and the liquid culture medium filtrate were used as controls.

The qualitative analysis of glycosidase was estimated by *p*-nitrophenol released in culture solutions. The culture filtrates were compared with those controls, and then the yellowish culture filtrates were estimated as positive.

Effect of Culture Filtrates on Groups A, B, and H Red Blood Cells—Shake cultures of isolates in a liquid culture medium containing 20 g of glucose and 10 g of peptone per litre were grown at 25°C at a 165-rpm shaking rate.

On the 5th, 14th, and 21st day, culture filtrates were collected through a membrane filter (0.45 μm). Then the culture filtrates (0.8 mL) were mixed with a 10% A-, B-, and H-RBC saline solution (0.2 mL) and incubated at 25°C for 24 h. The reaction mixture was transferred to a piece of gauze, and the ABH blood group was determined by elution testing.

The elution tests were performed following Yada's method described above. Drops of blood were blotted on gauze and allowed to dry at room temperature overnight sheltered from direct sunlight. Threads of these bloodstains were used for the tests as controls.

Quantitative Analysis of Glycosidase in Culture Filtrate—Each 1.75μ*M* of substrates PNP-α-*N*-acetyl-*D*-galactosaminide, PNP-α-*D*-galactopyranoside, and PNP-α-*L*-fucopyranoside were dissolved in 0.4 mL of 0.01*M* potassium phosphate buffer (pH 6.0) and used as substrate solutions. After 30-min incubation at 37°C, the reaction was terminated by the addition of 0.6 mL of 0.4*M* sodium carbonate (Na_2CO_3), and the *p*-nitrophenol that was released was measured by its absorbance at 400 nm. One unit of glycosidase was found to release 1 nmole of *p*-nitrophenol in 30 min under these conditions.

Results and Discussion

Twelve genera of fungi, representing two Zygomycotina, six Ascomycotina, and twenty-four Deuteromycotina were isolated from the sample (Table 1).

Of these twelve genera, three species, *Cunninghamella elegance (C. elegance)* (Zygomycotina), *Penicillium simplicissimum (P. simpl.)* (Deuteromycotina), and *Aspergillus niger (A. niger)* (Deuteromycotina) were used for the experiments described in this paper.

Initially, to understand better the effect of soil fungi on the blood cells A, B, and H antigens, the cultivation of fungi was attempted on A-, B-, and H-RBC, following the slide cultivation method. After cultivation of 3 strains on RBC for 14 days, each culture medium became brownish and covered with mycelium. ABH types of RBC culture media were determined by an elution test using monoclonal anti-A, B, and H antibodies. Table 2 indicates that the A and B activities were almost completely lost, whereas the H activity remained unchanged. As controls, ABH types of *C. elegance, P. simpl.*, and *A. niger* were examined by elution tests using monoclonal antibodies. But from each sample of three strains, A, B, and H antigens could not be detected in this procedure. Then, a qualitative analysis of glycosidase from fungi was done by using *p*-nitrophenyl (PNP)-α-*N*-acetyl-*D*-galactosaminide, PNP-α-*D*-galactopyranoside, PNP-α-*L*-fucopyranoside, and PNP-β-*N*-acetyl-*D*-gluco-

TABLE 1—*Fungi identified from sample soil.*

Taxa	Number of Isolates
Zygomycotina	
Cunninghamella	1
Gongronella	1
Ascomycotina	
Eupenicillium (Penicillium)[a]	2
Neosatorya (Aspergillus)[a]	2
Talaromyces (Penicillium)[a]	1
Hamigera (Penicillium)[a]	1
Deuteromycotina	
Penicillium	15
Aspergillus	5
Fusarium	1
Trichoderma	1
Paecilomyces	1
Verticillium	1
Total Number of isolates	32

[a]Anamorphic state.

TABLE 2—*Elution tests from RBC culture media.*

Strain	RBC Types	Monoclonal Antibodies[a]		
		Anti-A	Anti-B	Anti-H
C. elegance	A	−	−	+
	B	−	−	2+
	H	−	−	2+
P. simpl.	A	−	−	+
	B	−	−	+
	H	−	−	2+
A. niger	A	−	−	+
	B	−	−	+
	H	−	−	2+
Control	A	3+	−	−
	B	−	3+	−
	H	−	−	3+

[a]3+ = the strongest agglutination,
2+ = strong agglutination,
+ = weak agglutination, and
− = no agglutination.

saminide as the substrate. The fungi were cultured in the peptone solution added to each of these substrates at 25°C with a 165-rpm shaking rate. The results reveal that α-*N*-acetyl-*D*-galactosaminidase, α-*D*-galactosidase, and α-*L*-fucosidase were produced from each of these strains, but the production of β-*N*-acetyl-*D*-glucosaminidase was not apparent (Table 3).

As the production of the A-, B-, and H-decomposing enzymes in these isolates was observed by this qualitative analysis, the relationship between activity of enzymes and this decomposition capacity was examined. Culture filtrates were collected from the shake culture

TABLE 3—*Qualitative analysis of fungal enzymes that decompose group specificities of blood group substances.*

Enzymes	*C. elegance*	*P. simpl.*	*A. niger*
α-*N*-acetyl-*D*-galactosaminidase	±	+	++
β-*N*-acetyl-*D*-glucosaminidase	±	±	±
α-*D*-galactosidase	+++	+++	+++
α-*L*-fucosidase	+++	+++	++

media on the 5th, 14th, and 21st day. The activities of the A-, B-, and H-decomposing enzymes in these filtrates were measured by quantitative analysis (Table 4). Then the effect of the filtrates on RBC was examined by an elution test using a monoclonal anti-A, B, and H antibody (Table 5).

A-decomposing enzymes produced by three strains, *C. elegance, P. simpl.*, and *A niger*, did indicate α-*N*-acetyl-*D*-galactosaminidase activity (Table 4). Treatment of group A erythrocytes with each of these filtrates showed an almost complete loss of agglutinability by the monoclonal anti-A antibody and consistently increased their reactivity with the monoclonal anti-H antibody.

This result shows close agreement with the behavior of the A-decomposing enzyme in the human and the pig liver [*29*], *Trichomonas foetus* [*17*] and *Helix pomatia* [*30*]. The A-decomposing enzyme, α-*N*-acetyl-*D*-galactosaminidase, released *N*-acetyl-*D*-galactosamine from the A substance, and the immunochemical A activity disappeared concominant with the development of H activity.

Alpha-*N*-acetyl-*D*-galactosaminidase is the enzyme that decomposes the blood group A determinant sugar. Two ways in which the decomposing mechanism of the A substance functions are known. One is by direct decomposition of *N*-acetyl-*D*-galactosamine by α-*N*-acetyl-*D*-galactosaminidase as seen in the human and the pig liver [*29*], *Trichomonas foetus* [*17*] and *Helix pomatia* [*30*]. The other is by decomposition of *N*-acetyl-*D*-galactosamine in two steps: first, by *N*-acetylase and, second, by *D*-galactosaminidase from *Clostridium tertium* [*7*]. There has been little study of purified *N*-acetylhexosamine deacetylase, which was seen to be inactive against phenyl-*N*-acetylglucosaminidases [*31*]. Thus, it may be considered that the A-decomposing enzyme in fungi is of the former type.

TABLE 4—*Enzyme activities of culture filtrates.*

Strain	Days	α-*N*-Acetyl-*D*-Galactosaminidase, U	α-*D*-Galactosidase, U	α-*L*-Fucosidase, U
C. elegance	5	8.8	2.9	12.0
	14	1.5	1.1	1.6
	21	1.5	<	<
P. simpl.	5	26.9	6.25	10.3
	14	14.9	12.8	14.5
	21	12.0	64.1	9.1
A. niger	5	7.9	132.0	13.5
	14	2.25	33.9	1.8
	21	3.6	13.0	2.4

[a] < = below the measurement range.

TABLE 5—*Elution tests of culture filtrates treated red blood cells.*

Strain	Days	RBC Types	Monoclonal Antibodies[a] Anti-A	Anti-B	Anti-H
		A	—	—	2+
	5	B	—	2+	+
		H	—	—	2+
		A	—	—	2+
C. elegance	14	B	—	2+	+
		H	—	—	2+
		A	—	—	2+
	21	B	—	2+	+
		H	—	—	2+
		A	—	—	2+
	5	B	—	—	+
		H	—	—	2+
		A	—	—	2+
P. simpl.	14	B	—	—	+
		H	—	—	2+
		A	—	—	2+
	21	B	-	-	2+
		H	—	—	2+
		A	—	—	2+
	5	B	—	—	2+
		H	—	—	2+
		A	—	—	2+
A. niger	14	B	—	+	2+
		H	—	—	2+
		A	—	—	2+
	21	B	—	+	2+
		H	—	—	2+
		A	3+	—	—
Control		B	—	3+	—
		H	—	—	3+

[a]3+ = the strongest agglutination,
2+ = strong agglutination,
+ = weak agglutination, and
— = no agglutination.

B-decomposing enzymes produced by *C. elegance, P. simpl.*, and *A. niger* were seen to contain α-*D*-galactosidase activity (Table 4). The level of α-*D*-galactosidase decreased with age for *C. elegance* and *A. niger* but increased for *P. simpl.*

Producing patterns of glycosidases are generally different among the species or strains under the same cultivation conditions.

Differences of cultivation conditions (temperature, medium, and so on) influence that pattern of same species or strain. So, it was suggested that differences of the producing patterns of α-*D*-galactosidase occurred among the cultivation of three strains (Table 4).

The treatment of group B erythrocytes with all of the *P. simpl.* filtrates was almost total loss of B activity and an increase in H activity. After treatment of the group B erythrocytes with all of the *C. elegance* filtrates, the B activity remained unchanged and the H activity increased. In the case of *A. niger,* group B erythrocytes treated with the filtrate contained 132.0 (U) activity of the B-decomposing enzyme, and there was complete loss of B activity and an increase in H activity. But, in the other two filtrates [B-decomposing activity was 33.9

and 13.0 (U)] of group B erythrocytes, the B activity remained and the H activity increased. Thus, a B-decomposing enzyme (α-*D*-galactosidase) can act on a B substance to split *D*-galactose and be accompanied by the development of H activity. In the case of low B-decomposing enzyme activity, it can be assumed that the α-*D*-galactosidase acts on B-RBC incompletely. The same observation was made after treatment with an α-*D*-galactosidase preparation from coffee beans [*32,33*] or *Trichomonas foetus* [*16*].

Alpha-galactosidase from *Aspergillus niger* causes the complete hydrolysis of methyl-α-*D*-galactopyranoside and it also liberates 37 to 40% of the α-linked galactose residues from large polysaccharides such as galactomannans isolated from guar and locust beans [*34*].

Thus, it may be considered that two filtrates (α-*D*-galactosidase activity was 33.9 and 13.0 U) of *A. niger* could not decompose B activity completely because of substrate specificities.

B-decomposing capacity of α-*D*-galactosidase from *P. simpl.* at Days 5 (6.25 U) and 14 (12.8 U) was stronger than that of *A. niger* at Days 14 (33.9 U) and 21 (13.0 U) (Tables 4 and 5). So, between *P. simpl.* and *A. niger*, there may be some differences of α-*D*-galactosidase specificities.

H-decomposing enzymes produced by *C. elegance*, *P. simpl.*, and *A. niger* did contain α-*L*-fucosidase activity (Table 4). Treatment of group H erythrocytes with all of the filtrates remained unchanged by H activity. The specificity of the α-*L*-fucosidase from the isolates may be such that it is incapable of splitting the α-fucosyl linkage.

Further work is needed to examine the biochemical characterization of A-, B-, and H-decomposing enzymes.

In the present study, the author examined the glycosidases produced by soil fungi and the effects of these enzymes on human blood groups. Production of A-, B-, and H-decomposing enzymes and the effect on the RBC are compared among three strains from soil fungi; *P. simpl.* produced blood group A- and enough B-decomposing enzyme to destroy A and B substance with an increase in H activity. *A. niger* and *C. elegance* produced an A-decomposing enzyme, which showed an almost complete loss of blood group A activity. They also contained an α-*D*-galactosidase and an α-*L*-fucosidase, which incompletely destroyed the blood group B specific activity, but did not influence the blood group H specific activity.

In view of the results, the effect of soil fungi is very important in cases of determination of the blood group from blood soaked in soil.

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Address requests for reprints or additional information to
Shin-ichi Kubo, M.D.
Department of Legal Medicine
Nagasaki University School of Medicine
12-4 Sakamoto-machi
Nagasaki 852, Japan