Galactosidase Action on Human Blood Group B Active Escherichia coli and Ox Red Cell Substances

Abstract. *The blood group B specificity of* Escherichia coli O_{86} *antigen, a B specific oligosaccharide fraction from it, and the B activity of a preparation of an "infectious mononucleosis receptor" from cattle erythrocytes are destroyed by α-galactosidase from the coffee bean, as demonstrated for the* E. coli *antigen by three methods. Blood group H(O) activity appears simultaneously. Galactose is the only hexose released from all three substances.*

The specificity of the human blood group A and B glycoproteins, and probably also that of H(O) substance, is associated with oligosaccharide side chains on the blood group-specific macromolecules (*1*). Zarnitz and Kabat (*2*) and Watkins *et al.* (*3*) have shown that α-galactosidase from coffee beans splits galactose from human blood group B substance with the concomitant destruction of blood group B activity and the appearance of blood group H(O) specificity in the hemagglutination inhibition test. Substances possessing human blood group B specificity are also found in animals and microbes; thus *Escherichia coli* O_{86} (*4*) and the "infectious mononucleosis receptor" (IM-antigen) (*5*) of beef erythrocytes contain structures of high and moderate blood group B activity, respectively. The blood group B specificity of an *E. coli* O_{86} polysaccharide preparation determined with chicken antiserum to *E. coli* O_{86} was reportedly destroyed by an enzyme preparation from *Clostridium maebashi* with the liberation of galactose and oligosaccharides (*6*). A lipopolysaccharide from *E. coli* O_{86} (LPS-O_{86}) with blood group B activity has been obtained in highly purified and highly active form (*7, 8*), and several B active oligosaccharides have been isolated from it by mild acid hydrolysis and then partially characterized (*7*).

We have now investigated whether galactosidase from coffee bean induces biological and chemical changes on the LPS-O_{86}, on a blood group B specific oligosaccharide fraction isolated from it, and on the B cross-reactive IM-antigen similar to those on human blood group B substance. The effect of this enzyme on a blood group H(O) active polysaccharide from *Sassafras albidum* (*7, 9*), whose serologic specificity is determined by 3-*O*-methyl-D-galactose, was also studied.

Crude galactosidase was prepared from green coffee beans (*10*) with slight modification of the procedure of Helferich and Vorsatz (*11*) and partially purified by dialysis (*2, 3*). A fresh solution, apparently α-specific only, was prepared for each experiment, and it contained approximately 360 μg of nitrogen per milliliter, as determined by the Dumas method. All enzyme experiments were carried out under sterile conditions at a final *p*H of 5.2 to 5.4 (0.05*N* acetate buffer). One part of enzyme was incubated with four parts substrate (by weight) and with a few drops of toluene at 37°C for 4 to 5 days. The samples were shaken occasionally. The macromolecular substrates were used after thorough dialysis. The oligosaccharide preparation (fraction PA-O) contained two galactose, one fucose, two hexosamine, and two or three glucose units (*7*). As controls, the following were included: substrate with autoclaved enzyme (20 minutes at 120°C); and for the macromolecular substances, an additional control containing water instead of the enzyme solution. After incubation, the *p*H of each sample was adjusted to 6.8 to 7.0 with NaOH, and the mixture was then heated for 20 minutes in a boiling water bath. Portions were dialyzed at 4°C for 48 hours against 80 volumes of water containing a small amount of toluene; the dialyzate was changed three times. The specimens containing oligosaccharide substrate were dialyzed directly. The dialyzates were then concentrated in a vacuum and desalted with ion-exchange resin (MB-3). All materials were adjusted to appropriate concentrations for serologic tests and chromatographic analysis.

We attempted to inhibit the action of the galactosidase on LPS-O_{86} by D-galactose, the product of its own activity, since such an inhibition has been observed for other enzymes that inactivate blood group substances (*12*). D-Glucose was included as a control. A 20-fold excess (by weight) of monosaccharide over substrate was used in these experiments which otherwise were carried out as were the ordinary enzyme studies with macromolecular substrates.

Hemagglutination inhibition tests were performed as described elsewhere (*4, 13*); 0.1 ml of selected "immune" human serum, appropriately diluted (Table 1), was added to 0.1 ml of inhibitor serially diluted (twofold). The anti-H(O) agglutinins were those used earlier (*13*). The mixtures were shaken and left 2 hours at 22° to 25°C, and 0.1 ml of a 0.5 percent suspension of erythrocytes was then added; the mixtures were again shaken and read microscopically after incubation for 85 minutes at 22° to 25°C. Each titration series included the controls already described (*4*). All serological tests were performed at least three times for each sample with the exception of the tests with the "oligosaccharide," which were done only twice. The enzymatic and serologic results are listed in Table 1. They represent the average of three experiments except for the "oligosaccharide" where there was only one experiment. The blood group B activity of the *E. coli* O_{86} lipopolysaccharide is decreased by more than 94 percent. Blood group H(O) activity is produced anew as result of galactosidase action when measured with the eel anti-H(O) serum; it was not demonstrable with rabbit

Table 1. Inhibition of antihuman blood group B and H(O) agglutinins by blood group cross-reactive substances.

Blood group active material		Treatment	Minimum amount of material (mg/ml) giving complete inhibition of 4 agglutinating doses serum		
Group	Substance		Human anti-B*	Eel anti-H(O)	Human anti-A
B	*E. coli* O_{86}, lipopolysaccharide	None	0.005	>10	5–>10
		Galactosidase	0.1	1.2–2.5	>10
B	*E. coli* O_{86}, PA-O "oligosaccharide"	None	1.2–2.5	>10	>10
		Galactosidase	>10	5	
B	I.M.-substance from beef erythrocytes	None	1.2	>5†	>10
		Galactosidase	5	>5†	>10
O	*Sassafras* polysaccharide XII	None	>10	0.01	>10
		Galactosidase	>10	0.01	>10

* From A_1 and O individuals. † For reaction with *Cytisus* reagent see text.

anti-H(O) serum (one test only). Similarly, the group B activity of "oligosaccharide" PA-O was destroyed by the enzyme with the appearance of H(O) activity as tested with eel serum. The B cross-reactivity of the IM-antigen was less readily inactivated in that the inhibitory titer was regularly lowered by two tubes—that is, by 75 percent only. No inactivation of the highly H(O) active *Sassafras* polysaccharide was observed. The action of the galactosidase on LPS-O_{86} was specifically and completely inhibited by D-galactose, but there was no inhibition by D-glucose.

Erythrocytes may be sensitized by blood-group specific bacterial antigens and specific agglutinations obtained with properly absorbed antisera (*8*). The averages of four experiments on the action of group O erythrocytes, sensitized under standard conditions (*8*) with group B specific lipopolysaccharide before and after treatment with the galactosidase are shown in Table 2. Adsorptions and elutions of agglutinins with B cells by the Landsteiner-Miller procedure were carried out as described in an earlier report (*8*). Sera were absorbed twice. Absorption decreased the titer of the antiserum to group B erythrocytes from 1:256 to less than 1:1. The anti-B titer of the eluate was 1:32. Enzymatic inactivation of bacterial blood group B-like antigen was clearly demonstrable with eluted antibodies possessing B specificity (Table 2, bottom row). Even though the blood group B specific component is only a partial antigen of *E. coli* O_{86}, the enzymatic inactivation could also be shown with unabsorbed anti-B serum and, to a lesser extent, with the absorbed sera (second row). This effect, however, varied for different sera. It may indicate serologically important α-galactose determinants in *E. coli* O_{86} lipopolysaccharide other than those responsible for blood group B specificity.

Unabsorbed human "immune" antiserum to B substance gave one well-defined band with the untreated LPS-O_{86} in agar-gel diffusion (Ouchterlony's method) but no bands with the digested material. Lipopolysaccharide with high blood group H(O) activity from *E. coli* O_{128} gave one sharp band with eel anti-H(O) serum (*14*), but neither galactosidase-treated nor untreated lipopolysaccharide from *E. coli* O_{86} gave a visible reaction in this system. Neither was a cross-reaction with horse antiserum to pneumococcus type XIV demonstrable with the LPS-O_{86} before or after enzyme action. The IM-antigen did not react in any of the three systems under our experimental conditions.

Table 2. Reaction of agglutinins to blood group B with group O erythrocytes sensitized with the lipopolysaccharide from *E. coli* O_{86}.

Treatment	Reciprocal titer	
	Untreated	Enzyme-treated
Unabsorbed serum*	64	16
Serum absorbed with B cells	32	8–16
Eluate from B cells	8–16	1–2

* Same antisera to blood group B as in Table 1.

Paper chromatography of the dialyzable part of the digests uniformly showed the release of galactose from all B-active substrates tested. The oligosaccharide preparation PA-O revealed two new carbohydrate spots on chromatograms with an $R_{glucose}$ of 0.46 and 0.76, respectively [Whatman No. 1 paper; butanol, pyridine, and water (6:4:3)]. The migration rate 0.76 is closely similar to that of β-galactosyl (1→4)-*N*-acetyl-D-glucosamine. The component with $R_{glucose}$ 0.46 was considerably slower than α-D-galactopyranosyl (1→3)-D-galactose.

These enzymatic results furnish support for earlier immunological (*4*) and chemical (*7*) findings of the close relation between the human blood group B substance and the serologically active carbohydrate structures of *E. coli* O_{86}. The degree of destruction of the group B activity of LPS-O_{86} is of the same order as that obtained by Zarnitz and Kabat (*2*) for human blood group B substance. However, one difference is the appearance of considerably less H(O) activity than that found by Watkins, Zarnitz, and Kabat (*3*). The IM-antigen showed no H(O) activity either before or after galactosidase treatment when tested with eel serum. However, the treated material was active when an anti-H(O) extract from *Cytisus sessilifolius* was used. This observation may be explained by a linkage of the terminal galactose to a sugar different from that found in *E. coli* O_{86} or in human blood group B substance, since Watkins and Morgan (*15*) have shown that a different structure exhibits H(O) specificity with *Cytisus* extract than with the eel anti-H(O) serum. The blood group H(O) activity of the polysaccharide from *Sassafras albidum* was not enzymatically affected although the sugar possessing all the H(O) activity in the macromolecule is 3-*O*-methyl-D-galactose (*5, 19, 13*). Acid hydrolysis does not readily reduce the H(O) activity of the *Sassafras* polysaccharide, apparently because its structure consists of repeating units, and removal of surface structures uncovers similar ones (*16*). No carbohydrate structures involved in H(O) specificity were released by the enzyme from coffee bean, possibly because of the *O*-methyl group attached to the galactose, or because the linkage of 3-*O*-methyl-D-galactose to the macromolecule may be β, or because the sugar is present in the furanose form.

GEORG F. SPRINGER
JAMES H. NICHOLS
HUGH J. CALLAHAN
Department of Immunochemistry Research, Evanston Hospital Association, and *Department of Microbiology, Northwestern University, Evanston, Illinois*

References and Notes

1. V. P. Rege, T. J. Painter, W. M. Watkins, W. T. J. Morgan, *Nature* **200**, 532 (1963); G. Schiffman, E. A. Kabat, W. Thompson, *Biochemistry* **3**, 587 (1964).
2. M. L. Zarnitz and E. A. Kabat, *J. Am. Chem. Soc.* **82**, 3953 (1960).
3. W. M. Watkins, M. L. Zarnitz, E. A. Kabat, *Nature* **195**, 1204 (1962).
4. G. F. Springer, P. Williamson, W. C. Brandes, *J. Exptl. Med.* **113**, 1077 (1961); G. F. Springer, *J. Immunol.* **76**, 399 (1956).
5. G. F. Springer, in *The Amino Sugars*, R. W. Jeanloz and E. A. Balazs, Eds. (Academic Press, New York, 1964), chap. 46; G. F. Springer and H. Callahan, in preparation.
6. S. Yamamoto, T. Kogure, H. Ichikawa, S. Iseki, *Gunma J. Med. Sci.* **12**, 6 (1963).
7. G. F. Springer, J. H. Nichols, B. Kolecki, Abstracts, 147th Meeting Am. Chem. Soc., sect. C, p. 13 (1964). G. F. Springer, in *Immunbiologie*, 15th Symposium German Society of Physiological Chemistry, O. Westphal and H. Mayer-Kaupp, Eds. (Springer, Berlin, 1964), in press.
8. G. F. Springer and R. E. Horton, *J. Gen. Physiol.* **47**, 1229 (1964).
9. G. F. Springer, T. Takahashi, B. Kolecki, P. Desai, *Federation Proc.* **23**, 2 (1964).
10. Champion Coffee Co., Chicago, Ill.
11. B. Helferich and F. Vorsatz, *Z. Physiol. Chem.* **237**, 254 (1935).
12. W. M. Watkins and W. T. J. Morgan, *Nature* **175**, 676 (1955); G. F. Springer, "Immunochemistry of antigens," in *Immunohematology Colloquium*, H. Schubothe, Ed. (Springer, Berlin, 1956), p. 825.
13. G. F. Springer, P. R. Desai, B. Kolecki, *Biochemistry* **3**, 1076 (1964).
14. G. F. Springer, P. Williamson, B. L. Readler, *Ann. N.Y. Acad. Sci.* **97**, 104 (1962).
15. W. M. Watkins and W. T. J. Morgan, *Vox Sanguinis* **7**, 129 (1962).
16. P. Williamson and G. F. Springer, *Federation Proc.* **20**, 1 (1961).
17. Aided by NIH grant AI-05682 and by NSF grant GB-462. The Immunochemistry Department is maintained by the Susan Rebecca Stone Fund. The α-D-galactopyranosyl (1→3)-D-galactose was given by Prof. E. A. Kabat and the β-galactosyl (1→4)-*N*-acetyl-D-glucosamine by Dr. R. Tomarelli. The rabbit anti-H(O) serum was a gift from W. T. J. Morgan, and the horse antiserum to pneumococcus type XIV was given by Dr. J. L. Hendry. The coffee beans were donated by Mr. H. S. Tiny. The technical assistance of Miss R. Schuster, Mrs. H. Tegtmeyer, and Mrs. L. Schmidt is gratefully acknowledged.

7 July 1964