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BLOCKING OF HOG INTRINSIC FACTOR BY HUMAN GASTRIC JUICE AND CERTAIN MUCOPOLYSACCHARIDES, INCLUDING BLOOD GROUP SUBSTANCE *

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Intrinsic factor can bind vitamin B₁₂ and simultaneously attach to receptors on liver or small intestine in a calcium-dependent manner (1). There appears to be a stoichiometric relationship between the receptors, intrinsic factor and vitamin B₁₂ (2).

This report, previously presented in preliminary form (3), is concerned with blocking of the receptor sites for hog intrinsic factor by human gastric juice and certain mucopolysaccharides, including blood group substance.

MATERIALS AND METHODS

Gastric juice was collected before and after insulin stimulation of fasting patients. A Dreiling tube (4) was used to avoid contamination with saliva and bile.¹ Gastric juices were pooled according to the blood group of the donors, centrifuged, neutralized with NaOH (20 per cent), and frozen.

Saliva was collected from blood group A-secretors and filtered through gauze.

*Commercial preparations of blood group A and B substances*² were obtained from large pools of equine and porcine gastric mucosa after enzymatic digestion and alcohol precipitation.

Degraded blood group A and B polysaccharides ("P-1 fraction") were prepared from the commercial preparations (Merck) by mild acid hydrolysis with dilute HCl, pH 2.0, at 100° C for 2 hours, followed by dialysis for 4 days against 4 changes of tap water. This treatment splits off fucose and certain oligosaccharide side chains, some of which determine blood group specificity, but

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² Kindly provided by Doctor N. S. Ritter, Merck, Sharp and Dohme Research Laboratories, Rahway, N. J.

greatly increases cross reactivity with type XIV anti-pneumococcal horse serum (5, 6).

Hog intrinsic factor concentrate (HIFC), WES 727,³ was potent in a daily oral dose of 5 mg as measured by Schilling-type testing (7). It contained 15 to 20 per cent of blood group A activity as determined by the iso-hemagglutination technique (5). Twenty-five µg of this preparation was almost sufficient to saturate the receptors in a 10 ml aliquot of 10 g of rat liver homogenate suspended in 100 ml of Krebs-Ringer-Tris (KRT) medium (2). Other agents tested were: chondroitin sulfate (General Biochemicals, lot 26752); human chorionic gonadotropin ("Follutein," Squibb); heparin ("Liquaemin sodium," Organon); pneumococcus polysaccharides types I, VII, XIV and XVIII and Friedlander bacillus polysaccharide (obtained from Dr. Ely Perlman, Department of Bacteriology, The Mount Sinai Hospital, N. Y.); dextran, USP, 6 per cent wt/vol in saline (Abbott); hyaluronic acid powder, purity approximately 90 per cent (prepared by Dr. Boaz and provided by Dr. M. Horowitz, Department of Gastroenterology, The Mount Sinai Hospital, N. Y.); radioactive Co⁶⁰-vitamin B₁₂³ diluted to 10⁻⁸ g per ml, specific activity 1 µc per µg, which re-

TABLE I

*Inhibition of hog intrinsic factor concentrate (HIFC) enhancement of liver homogenate uptake of Co⁶⁰-B₁₂ by A, B or O pooled human gastric juice (HGJ) or A-secretor saliva **

First incubation Added agent	Second incubation Added agent	Counts/ minute	% Inhibi- tion
Experiment A			
25 µg HIFC plus:			
1 ml 0.9% NaCl	Co ⁶⁰ -B ₁₂	2,575 ± 261	
1 ml A-HGJ	Co ⁶⁰ -B ₁₂	612 ± 76	83
1 ml B-HGJ	Co ⁶⁰ -B ₁₂	574 ± 36	84
1 ml O-HGJ	Co ⁶⁰ -B ₁₂	395 ± 63	89
Experiment B			
25 µg HIFC plus:			
1 ml 0.9% NaCl	Co ⁶⁰ -B ₁₂	3,162 ± 182	
1 ml A-secretor saliva	Co ⁶⁰ -B ₁₂	615 ± 50	81

* Vitamin B₁₂ content of pooled HGJ and A-secretor saliva [*Escherichia coli*, Z strain (8); assay kindly performed by Dr. Herman Baker]. A-HGJ = 76 µg/ml; B-HGJ = 35 µg/ml; O-HGJ = 66 µg/ml; A-secretor saliva = 40 µg/ml. Approximate concentration of blood group substance in HGJ by iso-hemagglutination technique (5): A-HGJ = 70 µg/ml; B-HGJ = 30 µg/ml.

³ Kindly provided by Dr. L. Ellenbogen and Dr. W. L. Williams, Lederle Laboratories, Pearl River, N. Y.

TABLE II
Inhibition of HIFC enhancement of liver uptake of Co⁶⁰-B₁₂ by purified A and B blood group polysaccharides

First incubation Added agent	Second incubation Added agent	Third incubation Added agent	Counts/minute	% Inhibition
Experiment A				
25 µg HIFC plus: 0.9% NaCl	Co ⁶⁰ -B ₁₂		2,876 ± 18	
25 µg A-bgp	Co ⁶⁰ -B ₁₂		1,073 ± 25	63
25 µg B-bgp	Co ⁶⁰ -B ₁₂		2,027 ± 16	31
Experiment B				
0.9% NaCl	25 µg HIFC	Co ⁶⁰ -B ₁₂	2,733 ± 264	
25 µg A-bgp	25 µg HIFC	Co ⁶⁰ -B ₁₂	461 ± 14	83
25 µg B-bgp	25 µg HIFC	Co ⁶⁰ -B ₁₂	1,169 ± 25	57

corded approximately 1 cpm per µµg in our well-type scintillation crystal.

Two, three or four sequential incubations in KRT, each followed by three washes, were performed in two ways: 1) the agent under study was incubated with liver homogenate *prior* to the addition of HIFC and, 2) the agent under study and HIFC were mixed together and then incubated with liver homogenate. Otherwise, the protocol was as previously outlined (2). After final incubation with Co⁶⁰-B₁₂ the radioactivity retained in the washed homogenate was assayed. All samples were run in duplicate. In the tables, the arithmetic mean of the duplicate samples is recorded, ± the actual deviation from the mean.

RESULTS

The tables record the liver homogenate uptake of Co⁶⁰-B₁₂ after pre-incubation with HIFC and the decrease of that uptake recorded as "per cent inhibition" due to the agents tested.

Pooled human gastric juice (HGJ) from A, B, and O individuals and saliva from a blood group

A-secretor each diminish the effect of HIFC on the uptake of Co⁶⁰-B₁₂ by rat liver homogenate (Table I).

Purified blood group A or B polysaccharides (bgp), either mixed with HIFC in equal concentration and then incubated with liver homogenate (Table II, Experiment A), or added before incubation with HIFC (Experiment B), reduced the effect of HIFC. This reduction was greater when the homogenate was exposed to bgp before exposure to HIFC.

Pooled human gastric juice from blood group A donors (A-HGJ) retains its ability to block the effect of HIFC after heating or dialysis (Table III).

This inhibitory effect of A-HGJ may be almost eliminated by suspending liver homogenate previously incubated with A-HGJ in ethylenediamene-

TABLE III
Inhibition of HIFC effect on uptake of Co⁶⁰-B₁₂ by heated or dialyzed pooled human gastric juice

First incubation Added agent	Second incubation Added agent	Counts/minute	% Inhibition
Experiment A			
Heated* A-HGJ plus 25 µg HIFC	Co ⁶⁰ -B ₁₂	95 ± 15	95
Unheated A-HGJ plus 25 µg HIFC	Co ⁶⁰ -B ₁₂	63 ± 1	97
0.9% NaCl plus 25 µg HIFC	Co ⁶⁰ -B ₁₂	1,928 ± 14	
Experiment B			
Nondialyzed A-HGJ plus 25 µg HIFC	Co ⁶⁰ -B ₁₂	905 ± 67	76
0.9% NaCl plus 25 µg HIFC	Co ⁶⁰ -B ₁₂	3,764 ± 152	
Experiment C			
Dialyzed† A-HGJ plus 25 µg HIFC	Co ⁶⁰ -B ₁₂	747 ± 0	76
0.9% NaCl plus 25 µg HIFC	Co ⁶⁰ -B ₁₂	3,095 ± 0	

* HGJ heated in boiling water bath at 100° C for 20 minutes.

† HGJ dialyzed at 4°C for 22 hours against 2 changes of 4 L of distilled water.

TABLE IV

Reversibility with incubation in EDTA or 0.9 per cent NaCl of the inhibitory effect of A blood group-containing pooled gastric juice on the effect of HIFC on liver homogenate Co⁶⁰-B₁₂ uptake

First incubation Medium, KRT Added agent	Second incubation Suspended in	Third incubation Medium, KRT Added agent	Fourth incubation Medium, KRT Added agent	Counts/minute
1 ml A-HGJ	10 ml 0.015 EDTA	25 µg HIFC	Co ⁶⁰ -B ₁₂	2,539 ± 93
25 µg HIFC	10 ml 0.015 EDTA	NaCl	Co ⁶⁰ -B ₁₂	46 ± 11
NaCl	10 ml 0.015 EDTA	25 µg HIFC	Co ⁶⁰ -B ₁₂	3,187 ± 28
1 ml A-HGJ	10 ml 0.9% NaCl	25 µg HIFC	Co ⁶⁰ -B ₁₂	2,902 ± 364
25 µg HIFC	10 ml 0.9% NaCl	NaCl	Co ⁶⁰ -B ₁₂	1,423 ± 156
25 µg HIFC	10 ml 0.9% NaCl	25 µg HIFC	Co ⁶⁰ -B ₁₂	3,525 ± 21

tetraacetate (EDTA) or 0.9 per cent NaCl at 37° (Table IV).

When A and B bgp were incubated with liver homogenate without Ca⁺⁺ in the medium (KRT, where Ca⁺⁺ was replaced by an equivalent millimolar concentration of Na⁺) and HIFC was added to a subsequent incubation, the HIFC effect was minimally depressed (Table V). Conversely, with Ca⁺⁺ present in the medium during the pre-incubation with bgp, the effect of subsequently added HIFC was markedly diminished (Table V).

When the homogenate was incubated in either EDTA or 0.9 per cent NaCl after incubation with A or B-bgp or pneumococcus polysaccharide type XIV, there was no inhibition of the effect of subsequently added HIFC (Table VI). The effect of HIFC itself, however, was still marked after incubation in 0.9 per cent NaCl but not after incubation in EDTA (Table VI).

Some dialyzable sugars (fucose, certain oligosaccharide side chains) are released from blood group polysaccharides by mild acid hydrolysis.

TABLE V

Calcium-dependence of the effect of blood group substance on liver homogenate Co⁶⁰-B₁₂ uptake after incubation with HIFC

First incubation Medium, KRT Added agent	Second incubation Medium, KRT Added agent	Counts/minute
100 µg A-bgp	25 µg HIFC	324 ± 1
100 µg B-bgp	25 µg HIFC	678 ± 85
0.9% NaCl	25 µg HIFC	2,836 ± 36
Medium, KRT(-Ca)		
100 µg A-bgp	25 µg HIFC	2,660 ± 30
100 µg B-bgp	25 µg HIFC	2,514 ± 102
0.9% NaCl	25 µg HIFC	3,963 ± 31

The nondialyzable residue (P-1 fraction) (5, 6) was resuspended in 0.9 per cent NaCl and tested (as "degraded bgp"). Less degraded bgp was needed to obtain the same "per cent inhibition" of HIFC than was obtained with intact bgp (Table VII). The decrease in Co⁶⁰-B₁₂ uptake was proportional to the increased amount of the "inhibitor."

Table VIII records the inhibition by 25 µg aliquots of 16 different polysaccharides of the HIFC effect on the Co⁶⁰-B₁₂ uptake of rat liver homogenate. A moderate inhibitory effect of relatively large quantities of Cohn fractions III and IV, and haptoglobin and gastric juice from patients with pernicious anemia, has also been demonstrated.

DISCUSSION

The ability of hog intrinsic factor concentrate to enhance Co⁶⁰-B₁₂ uptake by rat liver homogenate was inhibited *in vitro* by normal human gastric juice from A, B, or O donors, or saliva from an A-secretor. The inhibitory effect remains after heating or dialysis of blood group A human gastric juice. Inhibition is greater when the agent under study is pre-incubated with the liver homogenate before addition of HIFC rather than when it is mixed with HIFC and then added to the homogenate.

This suggests that the inhibitory agents act by attaching to the liver "receptors" for intrinsic factor, thereby blocking attachment of intrinsic factor to the receptors.

The blocking of the intrinsic factor receptors by the various agents studied appears to be calcium-dependent, and reversible by removing Ca⁺⁺ from the system. The various "inhibitors" did not damage the integrity of the liver "receptors"

TABLE VI

Reversibility with incubation in EDTA or 0.9 per cent NaCl of the inhibition by certain polysaccharides of the effect of HIFC on liver homogenate Co⁶⁰-B₁₂ uptake

First incubation Medium, KRT Added agent	Second incubation Medium	Third incubation Medium, KRT Added agent	Fourth incubation Medium, KRT Added agent	Counts/minute
25 µg A-bgp	10 ml 0.015 M EDTA	25 µg HIFC	Co ⁶⁰ -B ₁₂	2,627 ± 127
25 µg B-bgp	10 ml 0.015 M EDTA	25 µg HIFC	Co ⁶⁰ -B ₁₂	2,921 ± 353
25 µg PnS XIV*	10 ml 0.015 M EDTA	25 µg HIFC	Co ⁶⁰ -B ₁₂	2,752 ± 363
1 ml 0.9% NaCl	10 ml 0.015 M EDTA	25 µg HIFC	Co ⁶⁰ -B ₁₂	2,468 ± 12
25 µg HIFC	10 ml 0.015 M EDTA	0.9% NaCl	Co ⁶⁰ -B ₁₂	224 ± 12
25 µg A-bgp	10 ml 0.9% NaCl	25 µg HIFC	Co ⁶⁰ -B ₁₂	2,320 ± 72
25 µg B-bgp	10 ml 0.9% NaCl	25 µg HIFC	Co ⁶⁰ -B ₁₂	2,765 ± 173
25 µg PnS XIV	10 ml 0.9% NaCl	25 µg HIFC	Co ⁶⁰ -B ₁₂	2,816 ± 113
1 ml 0.9% NaCl	10 ml 0.9% NaCl	25 µg HIFC	Co ⁶⁰ -B ₁₂	2,944 ± 120
25 µg HIFC	10 ml 0.9% NaCl	0.9% NaCl	Co ⁶⁰ -B ₁₂	1,022 ± 72

* Pneumococcus polysaccharide type XIV.

as manifested by the fact that the receptors remained capable of again binding HIFC after removal of the inhibitor. The relative ease of removal of polysaccharide inhibitors as compared with HIFC, on incubation in 0.9 per cent NaCl, suggests a lesser specificity of these agents than of HIFC for liver receptors.

In accordance with the concept of molecular complementarity as the basis of specificity (9), the minimal blocking effect of certain polysaccharides and the strong blocking effect of others suggest that there is a specific moiety of polysaccharide nature involved in the binding of intrinsic factor to liver receptors.

It is possible that this moiety of intrinsic factor consists mainly of terminal β -galactosyl groups. This is suggested by the findings that the most active "blockers" are P-1 fractions prepared from blood group substances and type XIV pneumococ-

cus polysaccharide, all of which react strongly with type XIV antipneumococcal horse serum (5, 6), the reaction appearing related to the presence of terminal β -galactosyl groups (10). The blood group substances themselves are somewhat less effective as blockers than their P-1 fractions, and other pneumococcus polysaccharides studied are less effective than type XIV. The postulated β -galactosyl end-group configuration may not be identical for intrinsic factor derived from different species.

These studies leave intact the concept (11) that the ability to bind to both receptor and B₁₂

TABLE VIII

Inhibition of enhanced liver homogenate Co⁶⁰-B₁₂ uptake due to HIFC by polysaccharides

Agent*	% Inhibition
A-bgp	74
B-bgp	55
Degraded A-bgp ("P-1 fraction")	90
Degraded B-bgp ("P-1 fraction")	75
Pneum. polysaccharide Type XIV	65
Pneum. polysaccharide Type I	11
Pneum. polysaccharide Type XVIII	8
Pneum. polysaccharide Type VII	30
Pneum. polysaccharide "C"	4
Hyaluronic acid	5
Bacillus polysaccharide Friedlander	8
Human chorionic gonadotropin	6
Heparin	2
Chondroitin sulfate	13
Dextran	1
Agar ("Bacto-Agar," Difco; purified†)	5

* Twenty-five µg of agent and of HIFC mixed prior to incubation.

† By Dr. Shaul Kochwa, Dept. of Hematology, The Mount Sinai Hospital, N. Y.

TABLE VII

Inhibition of HIFC activity when mixed with intact or degraded blood group polysaccharides

Agent mixed with 25 µg HIFC	Amount*	Inhibition
	µg	%
Intact A-bgp	25	74
Intact B-bgp	25	55
Degraded A-bgp ("P-1 fraction")	25	90
Degraded A-bgp ("P-1 fraction")	10	75
Degraded A-bgp ("P-1 fraction")	5	56
Degraded B-bgp ("P-1 fraction")	25	85
Degraded B-bgp ("P-1 fraction")	10	65
Degraded B-bgp ("P-1 fraction")	5	54

* Amounts of degraded bgp are stated as the weight of intact bgp from which they were derived.

simultaneously may be unique to intrinsic factor. Preliminary experiments with aqueous extracts (12) of consecutive segments of rat small intestine indicate that such extracts may also act to block the intrinsic factor receptor sites on rat liver homogenate. This blocking action diminishes progressively in the more distally located segments and may be related to the presence of decreased quantities of polysaccharides. It has been demonstrated (13) that the average quantity of blood group substance in human intestine is less in extracts made from more distal segments of small intestine. Thus, the greater vitamin B₁₂ absorption from ileum (14) than from duodenum and jejunum may be related to a lesser quantity of intestinal receptor blocking materials in the lower small intestine.

The fact that HGJ does not enhance Co⁶⁰-B₁₂ uptake by liver homogenate as much as does HIFC suggests that the former may contain a variable amount of substances able to attach to receptors and thereby block intrinsic factor but unable then to bind B₁₂. One such substance may be degraded intrinsic factor. This possibility is strengthened by the observation that HGJ, degraded by heat, functions as a blocking agent. The blocking action of degraded or undegraded blood group substance and other compounds secreted into the alimentary tract may constitute the major difficulty in the *in vitro* assay of intrinsic factor in human gastric juice, and may in part explain why such assay is generally reliable only in achlorhydric patients (15), who would have reduced ability to degrade blood group substances and intrinsic factor.

Refractoriness of some patients with pernicious anemia to HIFC therapy has been ascribed to an antigen-antibody reaction; it was suggested (15) that a possible antigen might be blood group A substance, or another contaminant in HIFC, rather than intrinsic factor itself. The present studies indicate that the antigen could be a degradation product of HIFC or of blood group substance, or even type XIV pneumococcus polysaccharide. In this connection it should be noted that P-1 fractions prepared from A substance are *antigenic in A individuals*. Antibody (precipitin) to such fractions does not agglutinate erythrocytes or sensitize red cells to render them agglutinable by Coombs serum (6).

However, refractoriness to HIFC may not be due to antigenicity of hog intrinsic factor since it has been demonstrated that sera of some patients with pernicious anemia, who have never received HIFC, inhibit the activity of HIFC (16). Another factor to consider in the "refractory" case is that the oral administration of a poor B₁₂-intrinsic factor product may produce only a minimal hemopoietic response (17).

The question legitimately arises as to the general applicability of data obtained in a heterologous system, such as the present one (HIFC with rat liver homogenate). In this connection the following points should be made. 1) rat intrinsic factor has been successfully used in place of HIFC in our experimental protocol (2). 2) Species specificity does not appear to play a role in the phase of intrinsic factor action under discussion here. Recent evidence suggests that species specificity is not involved in the attachment of intrinsic factor to receptors, but rather in the subsequent release of vitamin B₁₂ from intrinsic factor by a presumed enzyme (12, 18).

These studies may have some relation to the statistical association of pernicious anemia and blood group A, although such relationship is highly speculative (19, 20).

SUMMARY

The enhancing effect of hog intrinsic factor concentrate (HIFC) on Co⁶⁰-B₁₂ uptake by rat liver homogenate receptors may be reduced ("blocked") by blood group substance, degraded blood group substance ("P-1 fractions"), type XIV pneumococcus polysaccharide, human gastric juice (from A, B, or O subjects; undegraded, dialyzed, or heated), and to a lesser extent by other polysaccharides. Blocking is Ca⁺⁺-dependent and EDTA-reversible, as is the effect of intrinsic factor. In accordance with the concept of molecular complementarity as the basis of specificity, it is suggested that intrinsic factor may have terminal β -galactosyl groups which link to receptors on liver (or small intestine), and that various mucopolysaccharides are effective as blockers of this linkage in proportion to the similarity of their end-group conformation to that of intrinsic factor.

The possibility is raised that human refractoriness to HIFC may be due in part to cross reaction

with antibody to P-1 fractions or type XIV pneumococcus polysaccharide.

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