Immunology, 1973, **25**, 621.

Human Blood Group Activity of Human and Canine Intestinal Glycolipids Containing Fucose

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(*Received* 29*th December* 1972; *accepted for publication* 27*th February* 1973)

Summary. A number of fucose-containing glycolipids (fuco-lipids), which are similar in composition to those of human normal and malignant gastrointestinal tissue, have been isolated from whole small intestines of individual dogs. Dogs from which these fuco-lipids were isolated fell into two types according to the qualitative sugar composition of their fuco-lipids. Glycolipids from type I dogs contained glucose, galactose, glucosamine, galactosamine and fucose, while those from type II dogs contained the same sugars but lacked galactosamine. Fucolipids isolated from type I and II dogs were tested for both canine blood group and human A, B, H and Le^a and Le^b blood group activity. At the concentrations tested, only human blood group A activity was found in significant amounts, and only in those fuco-lipids which contained galactosamine (type I dogs). Of the fuco-lipids with human blood group A activity, some had activity comparable to that of glycoprotein blood group substances, while others had lower, but significant, activity. These latter fuco-lipids also had marked chromatographic differences, indicating that they are of several different structural types, a finding similar to the A active glycolipids of human red cell stroma. None of the isolated intestinal fuco-lipids had canine blood group activity. A fuco-lipid with Le^a activity was also isolated in relatively large amounts from a normal human whole small intestine.

INTRODUCTION

Glycolipids with A and B blood group substance activity have been isolated from human gastrointestinal mucosae, and with the development of a carcinomatous change these have been found to be deleted and relaced by significant accumulations of glycolipids with H and Le^a activity (Hakomori and Jeanloz, 1964; Hakomori, Koscielak, Block and Jeanloz, 1967; Hakomori and Andrews, 1970). A glycolipid which was isolated from this cancerous tissue was called 'tumour glycolipid' and attempts to isolate this glycolipid from normal gastric mucosa were unsuccessful. The structure for this glycolipid was recently published and it was shown to be closely related to human blood group substances but lacking both Le^a and H activity (Yang and Hakomori, 1971).

In previous reports (Smith and McKibbin, 1971, 1972; Hiramoto, Smith, Ghanta, Shaw and McKibbin, 1973) a group of dog small intestinal fucose-containing glycolipids

was described which contained qualitatively and quantitatively the sugar composition of the human A, B, H, Le^a and Le^b glycolipid blood group substances and 'tumour glycolipid'. These fuco-lipids seem to represent an extremely heterogeneous group of compounds, some individual dogs having several different types of intestinal fuco-lipids. While some of these fuco-lipids can be isolated in amounts adequate for structural determination, others can be isolated in only trace amounts. Those present in trace amounts could accumulate and become quantitatively important in malignant change. In an attempt to ascertain the number of different types of fuco-lipids in dog small intestine, including those present in trace amounts, immunologic techniques have been useful in identifying these compounds and giving some insight into their structure. The blood group activity of these canine and human intestinal glycolipids was tested with a variety of canine and human antisera. The results of this study are given below.

MATERIALS AND METHODS

Preparations and analysis of the glycolipids

The glycolipid fractions used in this work were prepared from eleven individual canine and two human whole small intestines. The methods of isolation and chemical analysis have been described previously (Smith and McKibbin, 1972; Vance, Shook, and McKibbin, 1966; McKibbin, 1969). The human intestines were prepared exactly as the dog intestines except that an acetone precipitation was performed on the washed whole lipid extract. This was accomplished by reducing the lipid extract in chloroform to 100 ml and adding 7·2 volumes of acetone. This mixture was allowed to stand in the cold overnight and the supernatant was discarded. The discarded supernatant contained a maximum of 5 per cent of the lipid 'galactose' as measured by the anthrone method and 2 per cent of the lipid phosphorus. The glycolipids used for this study are identical to those described in a previous paper (Smith and McKibbin, 1972) except that fuco-lipids from one additional dog (dog 15F-1) and two humans (human 2F-1; human 3F-1) have been added to the series. The dog intestinal ceramide pentahexoside (Vance *et al.*, 1966) used in this study has been shown by Dr Sen-itiroh Hakomori to be immunlogically identical, by the Ouchterlony technique, to the Forssman hapten isolated from horse spleen. A partial structure for the dog intestinal Forssman hapten (Vance *et al.*, 1966) and the complete structure for horse spleen Forssman hapten have been proposed (Siddiqui and Hakomori, 1971).

The terms F-1 and F-2 refer to fuco-lipids isolated by silica gel G preparative thin-layer chromatography with a chloroform/methanol/conc. NH_4OH (40 : 80 : 25) solvent system (system B). The fuco-lipid with the greater Rf value was designated F-1, and the component with the lower Rf, F-2. An arabic number preceding this designation refers to the individual dog (e.g. 3F-1). The F-2 fuco-lipid in some dogs was further resolved into two components on silica gel G using a chloroform/methanol/H_2O/glacial acetic acid (55:45:5:5) solvent system (system D). The component with the higher Rf was designated F-2A, the component with the lower Rf, F-2B (Smith and McKibbin, 1972). The human fuco-lipids were separated on silica gel G thin-layer chromatography using system D. This system can be used rather than system B because the Forssman hapten is absent from human intestinal tissue. A human fuco-lipid is designated by 'Hu' (e.g. Hu-2F-1).

Immunological methods

Samples (200 μg) of purified canine glycolipids alone and with chromatographically

pure egg lecithin added in a ratio of 2 μM lecithin per μM glycolipid were individually taken to dryness under a nitrogen stream. Saline (2 ml) was added and the samples were shaken vigorously for 10 minutes at 60°. No enhancement of serological activity of the glycolipids was noted with the added lecithin, and it was omitted from further glycolipid preparations. Glycolipid samples dissolved in saline appeared clear and remained so for at least 24 hours. These preparations could be kept frozen for several months with no loss of serological activity. The canine glycolipids were tested for inhibitory activity against human and canine isohaemagglutinins, with concentrations of the glycolipid preparations at which inhibitory activity was detectable with purified human A, B and H substances, and with agglutinating systems capable of detecting blood group activity in 1:5 dilutions of human and canine saliva.

Two methods of detection were used: (a) for the detection of inhibition of naturally-occuring human anti-A and anti-B sera, of the *Ulex europaeus* anti-H agglutinin, and of the canine 'immune' isohaemagglutinins (Swisher and Young, 1961), the quantitative inhibition technique of Gibbs, Collins and Akeroyd (1961) was used, adapted to electronic particle counting by the aggregate-exclusion method of Bowdler and Swisher (1964a, b). Preliminary agglutination assays were made for each agglutinin, using 1 ml of a test red cell suspension with a cell concentration of 12,000 /μl, and 1 ml of serial dilutions of the antiserum or lectin. Suspensions and dilutions were made in isotonic phosphate-buffered saline at pH 7·4 containing 3 g per cent bovine serum albumin (Specific Bovine Albumin, Dade). Optimal conditions of temperature, sensitization time, centrifugation and disaggregation to obtain a system in equilibrium were determined individually for each system of agglutinin and red cells. The concentration of antiserum or lectin agglutinating 50 per cent of the cells of the standard suspension was determined by regression analysis of the probit-log relationship between percentage of cells agglutinated and the concentration of serum or lectin. The quantity of agglutination present in the standard volume at this concentration was adopted as the haemagglutinating unit (HD_{50}). Statistical methods were those of Emmens (1948). Screening tests of inhibition were made using 0·5 ml of a serum dilution containing 5 HD_{50} of serum or lectin, with the exception of the canine anti-C serum in which prozone phenomenon limited the quantity to 1 HD_{50}. To this were added 0·5 ml of a solution of the test substance at a concentration of 20 μg/μl, and 1 ml of the red cell suspension. Incubation, centrifugation and disaggregation were performed as before. In those instances in which significant inhibition was obtained a comparative agglutination inhibition assay was performed, using blood group substances as standards. Inhibition assays employed 0·5 ml of agglutinin containing 5 HD_{50} doses, 0·5 ml of diluted inhibitor and 1·0 ml of the standard suspension of red cells. A sequence of dilutions of inhibitor was used to estimate the amount of inhibitor required to reduce agglutination to 50 per cent, i.e. to inhibit 4 of the 5 HD_{50} of agglutinin present. In the present work, the haemagglutination inhibitory dose (HID) was defined as that quantity of substance required to inhibit 1 HD_{50} of agglutinin: the quantity required to reduce agglutination to 50 per cent is thus 4 HID. (b) For testing inhibition of human anti-H, from an O_h donor, anti-Le^a and anti-Le^b, conventional tube agglutination methods were used, employing 0·3 ml of antiserum, 0·2 ml of 2 per cent test cell suspension and 0·2 ml of a solution of the test substance in phosphate-buffered saline. After incubation for 30 minutes and centrifugation, agglutination was assessed microscopically on a scale of −, trace, ±, + through ++++.

Immunological materials

Naturally-occurring human anti-A and anti-B sera were obtained from human volunteers of blood groups B and A_1; human anti-H was obtained from a Bombay O_h subject (Pol.); immune canine antisera of specificities anti-A, -B, -C, -D, and -F were obtained from repeatedly immunized dogs; anti-H lectin was obtained from seeds of *Ulex europaeus* as a saline extract; anti-Le^a and anti-Le^b were obtained as commercial preparations (Dade, No. LEA-34AD and No. LEB-23AD).

Red cells were obtained from normal human subjects of blood types A Le^a, B Le^b, and O Le^b, and from a dog (Hd.) of canine blood type A_1BCDF.* Canine cells were freshly drawn on each occasion; human cells were stored in Alsever's solution for not longer than seven days before use.

Preparations of blood group substances were obtained from Dr Georg Springer of Northwestern University, Illinois, and included human A substance (CA 6961), human A substance prepared from meconium (CA 731), hog A substance (CA 1089), human B substance (CA 1185) and H substance (CA 1292).

Saliva was obtained from normal human subjects and from normal dogs. The saliva was centrifuged to remove debris, heated to 100° for 10 minutes and filtered (Millipore, 0·45 μM). Dilutions of 1 part saliva with 4 volumes of 0·9 per cent sodium chloride solution were used for inhibition studies.

RESULTS

YIELDS OF FUCO-LIPIDS AND RECOVERIES

The yields of isolated glycolipids are usually related to the weight of fresh tissue or dried lipid-free tissue. The intestinal tissue used in this study was usually fresh frozen and stored in this condition until extracted with organic solvents. Previous studies on pooled dog intestine (McKibbin, 1969) indicated that 'fresh tissue: dry lipid-free tissue' ratio was 6·85. The average value for 'frozen tissue: dry lipid-free tissue' for eleven individual dog small intestines was 6·52. The values reported here for fuco-lipid yields can thus be related to fresh tissue weight. This same ratio for human tissues was slightly higher at 7·58, although the small intestines of only two humans have been analysed. The yields of fuco-lipids from eleven dogs and two humans indicated that yields from human intestinal tissue were higher than those from dog.

Recovery of glycolipids by various isolation procedures was incomplete. The average recovery of total neutral sugar from dog tissue was 84 per cent from DEAE-cellulose columns and 73 per cent from preparative silica gel G thin-layer chromatograms. The total fuco-lipids isolated varied among individual dogs from approximately 4 to 90 μM of 'galactose' as measured by the anthrone reaction. There is a possibility that the concentration of intestinal fuco-lipids varies between individual dogs, but at present this cannot be precisely determined due to incomplete recoveries of total glycolipid.

VARIABILITY OF FUCO-LIPID COMPOSITION AND STRUCTURE

The results presented in Table 1 show that the glycolipids isolated from individual dog and human whole intestines all contained glucose, galactose, glucosamine and fucose.

* The canine typing systems carry designations which do *not* imply relationship to the similarly designated system of other species.

Fuco-lipids which have been isolated previously and shown to contain only these sugars have been designated type II (Smith and McKibbin, 1971, 1972). Dogs or humans from which fuco-lipids have been isolated that contained, in addition, galactosamine, have been designated type 1.

In general, if more than one fuco-lipid was isolated from an individual dog intestine, all the fuco-lipids either had galactosamine or lacked this sugar. The one exception appeared to be dog 8. At least three chromatographically different fuco-lipids were isolated from this dog. From paper chromatography glucosamine appeared to be present in higher concentrations than galactosamine (Smith and McKibbin, 1972). This dog has tentatively been designated type III, and it is possible that the fuco-lipids of this dog were a mixture of fuco-lipids with and without galactosamine. Quantitative analysis of the fuco-lipids of dog 8 was not possible owing to the small amounts isolated. Fuco-lipids from type I

TABLE 1.

MOLAR RATIOS, OR QUALITATIVE ANALYSES, OF SUGARS PRESENT IN FUCO-LIPIDS OF DOG AND HUMAN SMALL WHOLE INTESTINES

	Dog type	Fuco-lipid	Glu-cose	Galac-tose	Glucos-amine	Galactos-amine	Fu-cose	Total hexosamine
Dog No.								
1	II	F-1	+	+	+	0	+	
2	I	F-1	1·2	1·5	+	+	1·0	1·9*
3	I	F-1	+	+	+	+	+	
4	I	F-1	+	+	+	+	+	
5	II	F-1	1·0	1·5	0·84	0	0·81	0·84
6	II	F-1	1·0	1·7	1·4	0	1·7	1·4
8	III	F-1	+	+	+†	+	+	
8		F-2A	+	+	+†	+	+	
8		F-2B	+	+	+	+	+	
9	II	F-1	1·0	1·9	1·2	0	1·3	1·2
9		F-2	1·0	1·8	0·96	0	1·7	0·96
12	I	F-1	1·0	1·9	+	+	0·97	1·9*
12		F-2	+	+	+	+	+	
13	II	F-1	1·0	2·7	1·1	0	1·1	1·1
13		F-2	1·0	1·8	1·0	0	2·0	1·0
15	I	F-1	1·0	1·6	+	+	0·68	1·8*
Human No.								
2	II	F-1	1·0	2·2	1·1	0	0·83	1·1
3	I	F-1	1·0	+	+	+	+	

* Glucosamine and galactosamine appeared to be present in equal concentrations on paper chromatograms (Smith and McKibbin, 1972).

† Glucosamine appeared on paper chromatograms to be present in higher concentrations than did galactosamine (Smith and McKibbin, 1972).

dogs that have been isolated in large enough amounts to quantitatively estimate the sugars appeared to be ceramide hexaglycosides with glucose : galactose : hexosamine : fucose in the ratio of 1 : 2 : 2 : 1. Fuco-lipids isolated from type II dogs appeared to be either ceramide penta- or hexaglycosides with glucose : galactose : glucosamine : fucose in molar ratio of 1:2:1:1; 1:2:1:2; or 1:3:1:1. The fuco-lipid isolated from human 2 also had molar ratios of these sugars of 1:2:1:1, respectively. In this particular sample, traces of at least two other different compounds which migrated as fuco-lipids were detected on analytical thin-layer chromatography.

BLOOD GROUP ACTIVITY

The results of the quantitative inhibition survey of the glycolipids with human anti-A

are shown in Table 2. Inhibition is calculated as the percentage agglutination by which the test sample agglutination falls short of the lower 2 SD limit of the normal range, as determined in each case with four uninhibited control samples. 'Percentage inhibition' then expresses this deficit as a percentage of the mean agglutination obtained with the control samples.

TABLE 2

QUANTITATIVE INHIBITION SCREENING OF GLYCOLIPIDS WITH HUMAN ANTI-A AGGLUTININ

	Anti-A (10 μg)
Dose of inhibitor:	
Uninhibited agglutination per cent:*	85·1 (1·9)
Agglutination:	Human; natural
Dose of agglutinin:	5 HD_{50}
Test cells:	Human A_1
Inhibitor	Per cent Inhibition
Human A substance (CA696)	85·0
1F-1	5·5
3F-1	67·2
4F-1	64·3
5F-1	4·9
6F-1	2·4
8F-2A	11·8
8F-2B	14·3
8F-1	19·0
9F-2	1·6
9F-1	3·3
12F-1	50·1
13F-2	1·6
13F-1	2·2
15F-1	33·7
Hu-2	1·5
Forssman	7·3

* 2 SD value in parentheses.

TABLE. 3

COMPARISON OF INHIBITORY ACTIVITY OF CANINE GLYCOLIPIDS AND BLOOD GROUP SUBSTANCES IN A STANDARD INHIBITORY HUMAN ANTI-A SYSTEM WITH 5 HD_{50} OF AGGLUTININ

Substance	Slope of Probit-log inhibition curve*	Maximal inhibitory dose (μg)	Dilution giving 50 per cent agglutination	Quantity equivalent to 1 HID (μg)	HID/mg
3F-1	−1·13(0·11)	10	0·313	0·8	1280
4F-1	−1·31(0·16)	10	0·266	0·7	1500
12F-1	−0·93(0·12)	50	0·325	4·1	250
15F-1	−0·90(0·09)	50	1·000	12·5	80
A substance (CA696)	−0·81(0·04)	5	0·319	0·4	2500
A substance (CA731)	−1·17(0·03)	75	0·118	2·2	450
A substance (CA1098)	−1·12(0·07)	75	1·070	20·0	50

* 1 SD in parentheses.

With respect to A-activity, at least minimal traces of activity were present in all samples tested at the 10 μg level; the four most active were 3F-1, 4F-1, 12F-1, and 15F-1, and these

were subjected to formal comparative assays against the A-substances CA 696, CA 731 and CA 1089. The assays showed that the four canine substances had inhibitory activity comparable to the standard preparations of A substances. Table 3 gives data on the relative activity of the four most active canine preparations and the three standard preparations. The F-1 fuco-lipids of dogs 2, 3, 4, 12 and 15 had strongest human A activity and had similar chromatographic mobility in system D (Smith and McKibbin, 1972). Conditions of testing 2F-1 differed slightly from those later adopted and the data are not included in Table 2. The three fuco-lipids isolated from dog 8 (F-1, F-2A, F-2B) had weaker, but significant, human A activity (Table 2) but migrated with different Rfs on thin-layer chromatography. This finding suggested that dog intestinal fuco-lipids with human A activity may be polymorphic as has been reported for human red blood cell fuco-lipids with A and B activity (Hakomori and Strycharz, 1968; Koscielak, Piasek and Gorniak, 1970). All fuco-lipids with significant human A activity contained galactosamine; suggesting that galactosamine is the terminal residue on the glycolipid carbohydrate chain.

No significant inhibitory activity in the human anti-B system nor inhibitory activity comparable to human H substance was demonstrated for any of the fuco-lipids tested. Repetition of the experiments with a lower concentration of the Ulex preparation (3 HD_{50}) also gave no significant inhibition with 10 μg of canine glycolipids.

Tube agglutination studies of inhibition of human anti-H, anti-Le^a and Le^b showed inhibition of anti-Le^a by Hu-2F-1 but otherwise no inhibition at concentrations of antiserum readily inhibitible by 1:5 dilutions of the appropriate saliva. Although Hu-2F-1 was by far the major fuco-lipid isolated, and had Le^a activity, the blood type of this individual was A. Traces of other fuco-lipids were detected in this individual.

No significant inhibition was found with any of the systems tested using quantitative inhibition screening of the 'immune' canine blood group systems.

DISCUSSION

Intestinal glycolipids with human blood group activity

Although glycolipids have long been known to carry human ABH and Lewis blood group activity (Hakomori and Strycharz, 1968; Yamakawa and Iida, 1953; Yamakawa, Ota, Ichikawa and Ozaki, 1958; Yamakawa, Irie and Iwanaga, 1960; Yamakawa, Nichimura and Kamimura, 1965; Hakomori, 1954; Radin, 1957; Hakomori and Jeanloz, 1961; Handa, 1963; Koscielak, 1962; 1963), studies of chemical composition and structure have been difficult because of low yields of these substances. Additionally, variability of fuco-lipids which carry blood group activity among individual animals precludes pooling of tissues (Hakomori and Strycharz, 1968; Smith and McKibbin, 1971, 1972). Fortunately dog and human small intestinal tissue represents such an excellent source of these fuco-lipids that enough material can be isolated from a single animal to pursue structural studies. A report on pig intestinal tissue (Suzuki, Makita and Yosizawa, 1968) indicated that that tissue may also be a source of fuco-lipids structurally related to ABH and Lewis type glycolipids, but lacking any of these activities. The two fucolipids reported in that paper (PIGL-I and PIGL-II) are probably mixtures of fuco-lipids as were the two preparations (D-1 and D-2) isolated from pooled dog intestines (McKibbin, 1969). Since this work was done on pooled tissues and since pigs possess glycoproteins with human blood group A and H activity (Bendich, Kabat and Bezer, 1947; Watkins, 1966; Lloyd, Kabat, Layug and Gruezo, 1966; Carlson, 1968a, b), it is possible that further investi-

gation on individual pig intestines will produce a number of different fuco-lipids, some of which will carry human blood group activities. It is of interest that no evidence was found for a relationship between the dog fuco-lipids and the canine blood group activities, at least at the concentrations tested. This finding, along with the findings that some of the dog intestinal fuco-lipids had human A blood group activity and none had human B activity, recall the canine AXY digestive group tissue antigens reported by Zweibaum and co-workers (Zweibaum, Oudea, Halpern and Veyre, 1966; Zweibaum and Steudler, 1969a, b). Some of the AXY antigens had human A activity, none had human B activity, and they apparently were absent from dog erythrocytes. It is possible that dog intestinal fuco-lipids described in the present paper are identical to the AXY digestive group tissue antigens. Galactosamine is always present in those fuco-lipids having significant A activity and is absent from fuco-lipids lacking A activity. This evidence, along with the loss of galactosamine in periodate-treated intestinal glycolipids, suggests that galactosamine is terminal in the glycolipid carbohydrate chain. The thin-layer chromatographic differences in fuco-lipids with similar blood group activity isolated from dog intestine could be due to differences in the carbohydrate chain length as reported for polymorphic fuco-lipids of human red blood cell stroma (Koscielak *et al.*, 1970; Hakomori and Andrews, 1970). Accurate estimation of molar ratios of carbohydrates in glycolipids by colorimetric techniques is sometimes difficult. High fucose values by the cysteine-sulphuric acid method of Dische (1955) have been reported (Hakomori and Strycharz, 1968) and were experienced in our laboratory on mixtures of sugar standards in the molar ratios in which they occur in ABH and Lewis blood groups active glycolipids. These high values can be corrected by taking into account the absorbancies produced by glucose and galactose.

ACKNOWLEDGMENTS

The authors wish to thank Dr Sen-itiroh Hakomori for the Ouchterlony test on dog intestine Forssman hapten, Dr Georg Springer for preparations of blood group substances, and Mr Walter Shaw for the egg lecithin. We also thank Miss Deanna Fendley and Mr Walter Johnson for their technical assistance.

The work was supported in part by the following grants: USPHS NIH Grant 5-TO1-DE-7; NSF GB-15605; American Cancer IN 66K, and USPHS NIH Grant AM-12741.

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