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Monoclonal Antibodies Defining Blood Group A Variants with Difucosyl Type 1 Chain (ALe^b) and Difucosyl Type 2 Chain (ALe^y)†

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ABSTRACT: Three hybridomas secreting monoclonal antibodies, HH1, HH2, and HH3, defining different difucosyl A structures (ALe^b or ALe^y), have been established. Antibody HH1 (IgG2a) reacts specifically with the difucosyl A structure irrespective of a type 1 or type 2 chain, while antibody HH2 (IgG3) reacts exclusively with the difucosyl type 2 chain A (ALe^y) and does not react with the difucosyl type 1 chain or monofucosyl type 2 chain. Antibody HH3 (IgG2a) reacts exclusively with the difucosyl type 1 chain A (ALe^b) and does not react with the monofucosyl type 1 chain A or mono- and difucosyl type 2 chain A. These hybridoma antibodies were obtained by immunization of mice with purified glycolipid antigens and were selected by their reactivity with the specific glycolipid structures. These antibodies, together with previously established monoclonal antibody AH-21, specific for monofucosyl type 1 chain A, and monoclonal antibody TH-1, specific for type 3 chain A, are extremely useful to define blood group A variants present in cells and tissues.

The blood group A determinant is a well-established trisaccharide, GalNAcα1→3[Fucα1→2]Galβ1→R; however, the determinant is carried by a large number of core structures as listed in Table I [reviewed by Watkins (1980) and Hakomori (1981)]. With the recent development of the monoclonal antibody approach, the complexity and variation in blood group A determinants have been clearly established by specific monoclonal antibodies that distinguish among these A variants (Abe et al., 1984; Clausen et al., 1984, 1985a). It has become apparent that variation in core structure, but not in the A determinant itself, provides the basis for the presence of structurally and immunologically distinctive variants of A antigens, such as A_1 and A_2 (Moreno et al., 1971; Kisailus & Kabat, 1979; Clausen et al., 1985). Previously, we have established monoclonal antibodies AH-21 defining type 1 chain A (Abe et al., 1984) and TH-1 defining type 3 chain A (Clausen et al., 1985a). Among type 1 and type 2 chain A variants, two types of structures, monofucosyl and difucosyl, have been distinguished, as shown in Table I (1 and 2). Of particular interest is a wide distribution of difucosyl A determinants found in glycolipids of animal and human gas-

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Table I: Blood Group A Determinant Carried by Four Types of Carbohydrate Chains in Human Erythrocytes and the Monoclonal Antibody Defining Each Structure

	carbohydrate chain	monoclonal antibody
type 1 chain		
$A^{a\,a}$	GalNAcα1→3Galβ1→3GlcNAcβ1→3Galβ1→4Glc 2 ↑ Fucα1	AH21[b]
$ALe^{b\,a}$	GalNAcα1→3Galβ1→3GlcNAcβ1→3Galβ1→4Glc 2 4 ↑ ↑ Fucα1 Fucα1	this paper
type 2 chain		
$A^{a\,a}$	GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc 2 ↑ Fucα1	not established
$ALe^{y\,a}$	GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc 2 3 ↑ ↑ Fucα1 Fucα1	this paper
$A^{b\,c}$	GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc 2 ↑ Fucα1	not established
$A^{c\,c}$	GalNAcα1→3[Galβ1→4GlcNAc]$_1$β1 2 3 ↑ ↘ Fucα1 Galβ1→4GlcNAcβ1→3Galβ1→4Glc 6 ↗ GalNAcα1→3Galβ1→4GlcNAcβ1 2 ↑ Fucα1	not established
$A^{d\,c}$	the same as A^c, but the unit Galβ1→4GlcNAc in the β1→3 side chain is repeated twice, i.e., the number of the unit in brackets is two	not established
type 3 chain $A^{b\,d}$		
repetitive A	GalNAcα1→3Galβ1→3GalNAcα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc 2 2 ↑ ↑ Fucα1 Fucα1	TH-1[d]
type 4 chain $(A^x)^e$		
globo-A	GalNAcα1→3Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glc 2 ↑ Fucα1	not established

[a] Clausen et al. (1985b). [b] Abe et al. (1984). [c] Hakomori et al. (1972). [d] Clausen et al. (1985a). [e] Clausen et al. (1984). All these structures carrying A determinants are reactive with AH-16 (Abe et al., 1984).

trointestinal epithelia (Slomiany & Slomiany, 1975; Smith et al., 1975; Karlsson, 1976; McKibbin, 1978). A similar structure was previously found in human glycoproteins from blood group A individuals (Lloyd et al., 1966). The majority of A determinants in human erythrocytes are monofucosyl type 2 chain A, although the determinants are carried by unbranched (A^a, A^b) or branched (A^c, A^d) type 2 chain (Hakomori, 1981). In contrast, type 2 chain A (ALe^y) has been isolated and characterized from dog intestine (Smith et al., 1975), and difucosyl type 1 chain A (ALe^b) has been isolated and characterized from human intestine (McKibbin, 1978). In contrast to the predominance of type 2 chain in erythrocytes, type 1 chain A is preponderant in glandular tissues and secretions, although the proportion of monofucosyl/difucosyl A structure in various tissues is unknown. A small quantity of monofucosyl type 1 chain A defined by AH-21 has been found in erythrocytes of $Le^{a-b-c-d+}$ individuals (Clausen et al., 1985b), although the exact pattern of the distribution of these A variants in various tissues is unknown. Therefore, it is highly desirable to have a specific antibody for each of the A variants in order to study their distribution and possible changes associated with differentiation and oncogenesis. This paper reports the successful establishment of three monoclonal antibodies that specifically define difucosyl derivatives of type 1 and type 2 chain A determinants.

Materials and Methods

Antibody Preparation. The general procedure for establishing monoclonal hybridomas was followed according to a modification (Young et al., 1979) of Köhler & Milstein (1975). Purified glycolipids (0.5–2 μg) adsorbed on acid-treated *Salmonella minnesota* bacteria (50 μg) were used for intravenous immunization of BALB/c mice (3-month old), according to the schedule described previously (Young et al., 1979; Fukushi et al., 1984). Fusion with SP/2-0 (donated by Dr. C. Berglund, Fred Hutchinson Cancer Research Center, Seattle, WA) was made on the third day after the last injection. Screening of hybridomas was performed on 96-well plastic plates (Immunolon, Costar, Cambridge, MA) coated with 10–50 ng of glycolipids mixed with 50–250 ng of lecithin and 30–150 ng of cholesterol, as described previously (Kannagi et al., 1983a). Hybridomas were cloned by limited dilution

at least 3 times. Quantitative solid-phase radioimmunoassays were performed on 96-well, flat-bottom plates (Costar, Cambridge, MA) according to methods previously described (Kannagi et al., 1983a).

TLC immunostaining on high-performance thin-layer chromatography (HPTLC) plates was performed according to a modification (Kannagi et al., 1982) of the procedure of Magnani et al. (1980). Isotypes of the secreting immunoglobulin were determined by solid-phase radioimmunoassay using isotype-specific rabbit anti-mouse immunoglobulin antibodies (Young et al., 1979)).

Glycolipid Preparation. Blood type A blood was obtained from Puget Sound Blood Center (Seattle, WA) through the courtesy of Dr. Eloise Giblett and M. Osaki. Tumor tissue was obtained from NIH through the courtesy of Wilma Verrato of the Tumor Procurement Program of the National Institutes of Health. TH618, primary adenocarcinoma of liver, and FT418, liver adenocarcinoma metastasis from testes, were from blood group A individuals.

Glycolipids from erythrocyte membranes or tumor tissue were extracted in 2-propanol–hexane–water (55:25:20) as previously described (Kannagi et al., 1982), and the "upper phase glycolipids" were obtained by Folch's partition (Folch-Pi et al., 1951). The neutral glycolipid fraction was obtained by DEAE-Sephadex (A-25) chromatography (Yu & Ledeen, 1972). Purified glycolipid samples used in this study were prepared from the upper neutral glycolipid fraction by repeated fractionation by HPLC and subsequent HPTLC, both in native and peracetylated form. Glycolipids ALe^b and ALe^y were prepared from whole lipid extracts of human and canine intestine, respectively, by silicic acid and Florisil chromatography (Floridin Chemical Co., Tallahassee, FL) and solvent partition (Vance et al., 1966). The resulting mixture of complex native glycosphingolipids was dialyzed and resolved with preparative TLC (McKibbin, 1976; McKibbin et al., 1982). Each glycolipid fraction (ALe^b and ALe^y) was further purified as an acetate on HPTLC, and their structures were confirmed by ^{1}H NMR spectroscopy as previously described (Clausen et al., 1985b). Type 1 chain A^a was prepared from MKN-45 cell line as previously described (Abe et al., 1984). In addition, ALe^b and A^a type 1 chain were prepared from A blood as described elsewhere (Clausen et al., 1985b). Standard glycolipids A^a and A^b type 2 chain (Hakomori et al., 1974; Hakomori, 1981), A^x (Clausen et al., 1984), and A^b and A^c type 3 chain (Clausen et al., 1985a) were prepared from human erythrocytes. Forssman glycolipid antigen (Siddiqui & Hakomori, 1971) was prepared from goat erythrocytes. Le^b and Le^y (McKibbin et al., 1982) were prepared from canine and human intestine.

Results

Isolation of Hybridomas. Three hybridomas secreting antibodies specifically directed to difucosyl A determinants were isolated through a specific selection procedure with panels of defined glycolipids coadsorbed with lecithin and cholesterol on a multiwell plastic surface. The structures of glycolipids used for immunization and selection are shown in Table I. HH1 and HH3 were isolated after immunization of BALB/c with ALe^b glycolipid and were screened and selected for reactivity with ALe^b, ALe^y, or both. HH2 was isolated after immunization with ALe^y. The antibodies HH1 and HH3 were identified as IgG2a and HH2 as IgG3.

Specificity of Antibodies. The reactivities of established antibodies with various A determinants are shown in Figures 1 and 2. Figure 1 shows the reactivities of a constant, excess amount of glycolipids (10 μg/well) with different dilutions of

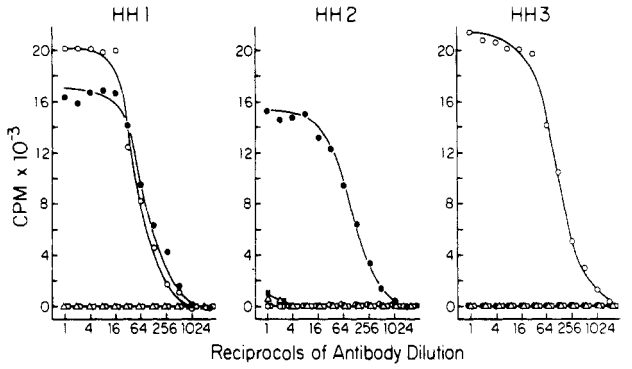

FIGURE 1: Antibody dilution. Reactivities of HH1, HH2, and HH3 with various glycolipid antigens at different concentrations of antibodies. The assay was made on Costar polyvinyl plates coated with 10 ng of glycolipid, 50 ng of lecithin, and 30 ng of cholesterol per well. Designations used were (○) A^{a}Le^b, (●) A^{a}Le^y, (△) A^a type 2 chain, (■) A^b type 2 chain, and (□) A^a type 1 chain. The initial concentration of antibody is the undiluted culture supernatant of hybridomas.

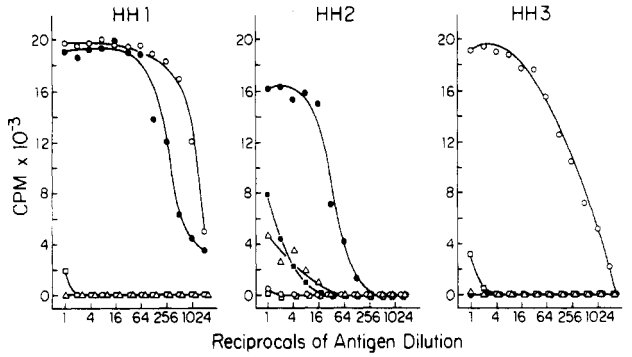

FIGURE 2: Antigen dilution. Reactivities of HH1, HH2, and HH3 with various glycolipid antigens at different concentrations of glycolipids. The initial concentration of glycolipids coated as in Figure 1 was 100 ng with 500 ng of lecithin and 300 ng of cholesterol. Undiluted hybridoma culture supernatants were used. Designations used were the same as for Figure 1. The glycolipids that gave no reactivity were A^x, A^b, and A^c type 3 chain, Forssman, Le^b, and Le^y. The concentration of antigen in the first well was 100 ng.

antibody. Figure 2 shows the reactivities of the undiluted hybridoma supernatants with varying amounts of glycolipids. HH1 showed strict specificity for the difucosylated A determinants irrespective of type 1 or 2 chain, whereas HH3 was specific for the difucosyl type 1 chain. HH2, in contrast, reacted only with the difucosylated type 2 chain A determinant. HH1 and HH3 showed no reactivity with other A determinants, whereas HH2 showed weak reactivity with monofucosylated type 2 chain A structures at high concentrations of both antigen and antibody. Antigen dilution curves with 1:10 dilution of the supernatants gave similar results for all antibodies; however, HH2 lost reactivity with the monofucosyl A determinants.

The specificities of the antibodies were further confirmed by inhibition of antibody binding to solid-phase glycolipids. The binding of antibody HH1 to ALe^b was inhibited by liposomes containing ALe^b and those containing ALe^y but not by liposomes containing type 1 chain A^a, type 2 chain A^a, or type 2 chain A^b. The binding of antibody HH2 to ALe^y was inhibited by liposomes containing ALe^y but not by those containing ALe^b, type 1 chain A^a, type 2 chain A^a, or type 2 chain A^b. The binding of antibody HH3 to ALe^b was only inhibited by liposomes containing ALe^b (See Figure 3).

Immunostaining on Thin-Layer Chromatography. For further confirmation of the specificities of the antibodies, immunostaining of the purified standards ALe^b, ALe^y, and

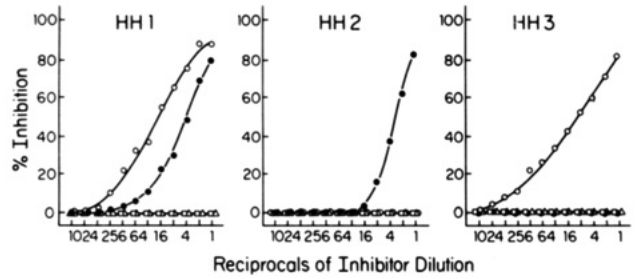

FIGURE 3: Inhibition of the reactivity of HH1, HH2, and HH3 by glycolipid liposomes. Plates were coated with 10 ng of glycolipid (HH1 and HH3, ALe^b; HH2, ALe^y) and 50 ng of lecithin and 30 ng of cholesterol. The initial concentration of the inhibitor glycolipid in liposomes was 1 μg/well. The concentration of the antibodies HH1 and HH3 was 1:30 of culture supernatant, and HH2 was 1:15. Designations were the same as in Figure 1.

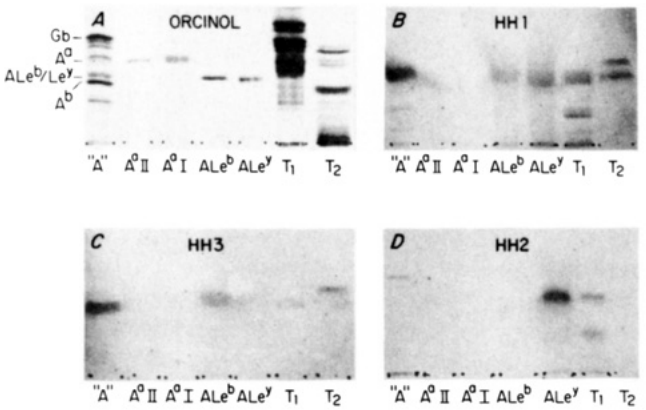

FIGURE 4: TLC immunostaining of glycolipid standards: (A) total upper neutral glycolipid from A erythrocytes; (T_1 and T_2) total upper neutral glycolipids of liver tumor tissue from blood group A individuals. Approximately 20 μg of total glycolipids were spotted; 1 μg of pure glycolipid standards type 2 A^a, type 1 A^a, ALe^b, and ALe^y were spotted. Panel A was visualized by orcinol/H_2SO_4 reaction; panels B–D were immunostained with HH1, HH3, and HH2, respectively. Plates were developed in chloroform–methanol–water (50:40:10). Note that HH2 reacted with the A^a region in the total glycolipids from erythrocytes but not the standard type 2 chain A^a (see Discussion).

A^a type 1 and type 2 chains separated on HPTLC was performed. In agreement with the reactivities observed by solid-phase assays using polyvinyl plates, HH1 stained both ALe^y and ALe^b, HH2 stained ALe^y, and HH3 stained ALe^b (Figure 4). HH2 did not stain the standard type 2 chain A^a, as expected; however, it stained weakly the A^a region of the total upper neutral glycolipid extract of A erythrocytes.[1] HH1 and HH3 staining of the ALe^b region in glycolipid extracts from A erythrocytes indicates the presence of ALe^b in erythrocytes (Figure 4B,C). HH2 did not stain glycolipids from A erythrocytes but stained the ALe^y/Le^b regions and a slower migrating band in the extract from tumor T_1 (Figure 4D) (see Discussion).

Discussion

Recent advances in glycolipid separation techniques by HPLC combined with mass spectrometry and NMR spectroscopy have enabled us to identify relatively small quantities of glycolipids present in highly complex mixtures [e.g., Kannagi et al. (1983b) and Clausen et al. (1984)]. Our ability to identify glycolipids has been greatly enhanced by the introduction of monoclonal antibodies defining specific carbohydrate sequences (Young et al., 1979). Previously, two types of A variants carried by type 1 and type 2 chain were distinguished [see Watkins (1980), Hakomori (1981) and Kabat (1982)]. Type 2 chain A determinant constitutes the major A determinant in human erythrocytes bound to either lipid (ceramide) or band 3 or band 4.5 protein (Fukuda et al., 1979). In contrast, type 1 chain A determinant is predominantly present in the glandular tissues and secretions of secretors but is absent in nonsecretors (Watkins, 1980). The presence of difucosyl type 1 chain A (ALe^b) in the erythrocytes of secretors with blood group Le^b (Le$^{a-b+c-d-}$) and monofucosyl type 1 chain A in erythrocytes of secretors with blood group Le^d (Le$^{a-b-c-d+}$) has been detected (Clausen et al., 1985b). In this previous work, we used monoclonal antibody AH-21, which defines monofucosyl type 1 chain A (Abe et al., 1984), and monoclonal antibody HH3, which defines difucosyl type 1 chain A (ALe^b) and is described in detail in this paper. Only with the application of these antibodies can we detect the presence of a small quantity of those type 1 chain glycolipids in the predominance of type 2 chain A present in erythrocytes, which have been overlooked by chemical analysis.

Three monoclonal antibodies, HH1, HH2, and HH3 as described in this paper, specifically react with difucosyl derivatives of type 1 and type 2 chain A structures and distinguish these structures from monofucosyl A structure. The specificities of these antibodies, HH1 for difucosyl A (ALe^b, ALe^y), HH2 for ALe^b, and HH3 for ALe^y, have been well established by solid-phase antibody binding assay, inhibition of antibody binding with glycolipid liposomes, and TLC immunostaining. These antibodies, together with previously established antibodies to type 1 chain A (Abe et al., 1984) and to type 3 chain A (Clausen et al., 1985a), have been proven to be extremely useful in the analysis of small quantities of A variants with mono- and difucosyl as well as type 1 and type 2 chain backbone. A specific location of blood group carbohydrate chains in the stratified architecture of epidermis as related to differentiation of the epidermis tissue has been established (Dabelsteen et al., 1982). These reagents are particularly useful in studying differentiation-dependent changes of oral epithelia. Furthermore, tissue-specific expression of A-variant structures may be reflected in primary tumors and metastasis. As shown in Figure 4B–D, "tumor 1" (T_1), a liver adenocarcinoma metastasis from testis, contained both ALe^b and ALe^y structures, while "tumor 2" (T_2), a primary liver adenocarcinoma, contained only the ALe^b structure. Another example of an application of these reagents is the analysis of A-like antigen present in tumors of blood group O and B individuals. Details of such A antigen expression in blood group O tumors will be described elsewhere (H. Clausen, S. Hakomori, N. Graem, and E. Dabelsteen, unpublished results).

Registry No. Difucosyl type 1 chain ALe, 98087-74-2; monofucosyl type 2 chain A, 30461-82-6; difucosyl type 2 chain ALe, 98087-75-3.

[1] TLC immunostaining of glycolipid mixtures is in some cases more sensitive than staining of pure glycolipids, possibly because of the carrier effect of phospholipid (Dr. Yoshio Hirabayashi, Shizuoka College of Pharmacy, Shizuoka, Japan, personal observation).

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Analysis of DNA Sequences Using a Single Chemical Cleavage Procedure

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ABSTRACT: A novel approach to sequence analysis of end-labeled, defined DNA fragments, using a single chemical cleavage procedure and electrophoretic separation in a single lane, has been developed. Prolonged treatment with hot aqueous piperidine results in partial cleavage of the DNA at all positions; the relative propensity for this cleavage is different for the various bases in the DNA. The hydrolysate is resolved on a DNA sequencing gel, and the distribution of radioactivity in the electrophoretic lane is analyzed (a) in terms of differential peak heights of the radioactive bands and (b) in terms of the spacings between successive bands. Simultaneous application of these two base-characteristic criteria allows the deduction of the nucleotide sequence with an accuracy approaching that of the established four-lane methods of DNA sequencing.

In the chemical cleavage method for DNA sequencing as developed by Maxam & Gilbert (1977, 1980), the DNA fragment of interest is provided with a radioactive tag specifically at one terminus and then subjected, in parallel, to *four* separate DNA cleavage procedures, which differ in their base specificities. Parallel resolution of the four reaction mixtures by polyacrylamide gel electrophoresis followed by visualization of the radioactive bands produces a four-lane pattern from which the nucleotide sequence of the fragment can be read off directly.

A considerable simplification would be achieved if the four parallel cleavage procedures were to be substituted by *one* cleavage protocol which should (a) be capable of severing the DNA backbone at *each* nucleotide position and (b) have distinct and characteristic propensities for DNA cleavage at the four different bases, adenine, guanine, cytosine, and thymine (A, G, C, and T, respectively).[1] The DNA sequence would then be deduced from the succession of radioactive bands of varying intensities in *one* electrophoretic lane. Such a method would also have good potential for automation of DNA sequencing.

The present is a report on a procedure which uses such differential cleavage at the various DNA bases, in conjunction

[1] Abbreviations: A, G, C, and T, adenine sites, guanine sites, cytosine sites, and thymine sites in DNA, respectively; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

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