Artificial Antigens. Antibody Preparations for the Localization of Lewis Determinants in Tissues†

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ABSTRACT: High-titer antisera specific for the Lewis-a, -b, and -d determinants were obtained by the immunization of rabbits with artificial antigens prepared by coupling chemically synthesized haptens to bovine serum albumin. The use of the appropriate synthetic immunoadsorbents allowed the isolation of the desired antibodies from the sera and the elimination of cross-reactive antibody populations. The resulting refined antibodies proved highly effective for the localization by immunofluorescence staining of Lewis antigens in epithelial cells of gastric and duodenal tissue of H patients. Furthermore, the immunohistochemical procedures could be performed in conventionally prepared paraffin tissue sections. In erythrocyte Lewis-a and -b individuals, the strongest staining reactions were with the appropriate reagent. Lewis-a individuals were Lewis-d negative. Tissue from Lewis-a–b– secretors showed strong positivity in the epithelial cells of the stomach only with the Lewis-d reagent while none of the reagents showed strong reactions with the tissue from the one nonsecretor Lewis-a–b– patient. The significance of these results to the biogenesis of the Lewis antigens is discussed.

Of the antigenic determinants that occur in human tissue, those of the blood-group-specific glycoproteins and glycosphingolipids are the best understood in terms of chemical structure, biosynthesis, and genetic transmission (Hakomori & Kobata, 1974). Since these substances are important histocompatability antigens (Wilbrant et al., 1969) and, in addition, are known to undergo changes both in neoplastic disease (Davidsohn, 1972; Hakomori et al., 1977) and aging (Hakomori et al., 1977; Race & Sanger, 1972), their distribution in tissues and body fluids has received much attention. Such studies have been handicapped by both the quality and the range of reagents available. Recent developments permit the chemical synthesis of significant portions of the oligosaccharide antigenic determinants for blood-group antigens (Lemieux et al., 1975, 1977). Thus, it is possible to prepare artificial antigens and immunoadsorbents that promise to be important for the improvement of the quality of antibodies specific for these determinants.

In this communication, we describe a new methodology for the preparation of antibodies for the specific detection of the Lewis-a (Le^a) (Rege et al., 1964), Lewis-b (Le^b) (Marr et al., 1967), and Lewis-d (Le^d) (Graham et al., 1976, 1978) blood-group determinants[1,2] and the application of these refined reagents in the immunohistochemical analysis of these antigens in the mucosa of the human stomach and duodenum of H blood-group individuals (see Table I). In addition, a number of preliminary observations are made with regard to the biogenesis of these antigens. Detailed consideration of their distribution and the morphological patterns observed in a larger population, which will include tissues from A, B, and AB individuals, are reserved for a separate communication (W. M. Weinstein, C. M. Switzer, R. Oriol, R. U. Lemieux, and D. A. Baker, in preparation).

Experimental Procedure

Preparation of Haptens. The haptens were synthesized as their 8-(methoxycarbonyl)octyl glycosides in order to facilitate the preparation of artifical antigens and immunoadsorbents. The preparations of the Le^a, Le^b, and Le^d determinants have already been reported [Lemieux et al., 1975, 1979; see also Lemieux (1978)]. The structures of the compounds were confirmed by detailed examinations of their ^1H and ^{13}C NMR spectra (Lemieux et al., 1980a).

Preparation of Artificial Antigens and Immunoadsorbents. The artificial antigens used for immunization of rabbits were conjugates of bovine serum albumin (BSA) and were prepared and analyzed as previously reported (Lemieux et al., 1975, 1977). The compositions of the artificial antigens were $[\alpha\text{LFuc}(1\rightarrow2)\beta\text{DGal}(1\rightarrow3)\beta\text{DGlcNAc}\rightarrow\text{O}(\text{CH}_2)_8\text{-CONH}]_{22}$BSA ($Le^d$ antigen), $[\beta\text{DGal}(1\rightarrow3)[\alpha\text{LFuc}(1\rightarrow4)]\beta\text{DGlcNAc}\rightarrow\text{O}(\text{CH}_2)_8\text{-CONH}]_{22}$BSA ($Le^a$ antigen), and $[\alpha\text{LFuc}(1\rightarrow2)\beta\text{DGal}[\alpha\text{LFuc}(1\rightarrow4)]\beta\text{DGlcNAc}\rightarrow\text{O}(\text{CH}_2)_8\text{-CONH}]_{37}$BSA ($Le^b$ antigen).

In order to collect the antibodies raised against the oligosaccharide-linking arm portion of these antigens, the hyperimmune sera were applied to affinity columns prepared from the corresponding hapten and aminated solid support following the previously described procedure (Boullanger et al., 1978) except that 100–120 mesh calcined diatomaceous earth was used instead of porous glass beads. The typical incorporations were in the range 0.4–0.7 μmol/g. The columns were washed with phosphate-buffered saline (pH 7.2) until free of unadsorbed protein and then bound antibodies were desorbed and eluted with 1% ammonium hydroxide. The antibody solution thus obtained was immediately neutralized and then dialyzed against phosphate-buffered saline and, thereby, also concentrated to the desired antibody concentration.

Immunization Protocols.[3] San Juan Rabbits were immunized with oligosaccharide BSA conjugates in Freund's

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[1] Although it is now established that, in terms of the αLFuc transferases that find expression in the formation of the Lewis antigenic determinants, the Lewis system would be properly delineated as $Le^a = Le^{a+d-}$, $Le^b = Le^{a+b+}$, $Le^d = Le^{a-b+}$, and $Le^c = Le^{a-b-}$, the conventional terminology for the Lewis phenotypes, namely, Le^a, Le^b, and Le^{a-b-}, will be used throughout this report. This procedure is adopted to avoid introducing, at this time, designations which may infer that the αLFuc transferase responsible for the development of the Lewis-d (H type 1) determinant is either different from or the same as that involved in the formation of the H (type 2) determinant.

[2] The phenotype Le^{a+b+} probably represents an extreme case of incomplete conversion of the Le^a antigen to Le^b (see Discussion).

[3] Animals used in this research were cared for in accordance with the principles contained in the Care of the Experimental Animals, a Guide for Canada, Canadian Council of Animal Care.

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complete adjuvant as previously reported (Lemieux et al., 1975). In all cases, the preimmune sera showed no reaction by immunodiffusion against the immunizing antigen. The blood was collected in vacutainer tubes and allowed to clot. The serum was separated from the clot and preserved by the addition of sodium azide to a final concentration of 0.02%.

Isolation and Refinement of the Antibodies. (*a*) *Anti-Lewis a.* Hyperimmune sera from six San Juan rabbits immunized with a Le^a–BSA conjugate were pooled (104 mL) and applied to an 18-g (1.6 × 20 cm) column of Le^a immunoadsorbent at 4 °C with a flow rate of 40 mL/h. Fractions of the eluate were collected (5.6 mL) and examined for the presence of anti-Le^a antibodies by immunodiffusion on agarose plates. Anti-Le^a antibodies were detectable in the eluate after ~85 mL of serum had passed through the column. The column was washed with phosphate-buffered saline (pH 7.2, containing 0.02% NaN_3, 250 mL) until the 280-nm absorption returned to base line. The bound antibodies were then desorbed at 4 °C with 1% ammonium hydroxide (35 mL) at a flow rate of 140 mL/h. The 280-nm absorbing eluate was immediately neutralized (pH 7.2) with a saturated solution of KH_2PO_4. The antibody solution was dialyzed against phosphate-buffered solution in an Amicon cell using an XM-50 membrane and concentrated to a final volume of 25 mL. After passage through a bacteria-excluding filter (0.2 μm pore size), the concentrated antibody solution (titer 7.3 mg/mL assuming $E^{1\%280} = 14$ for rabbit IgG) was examined by immunodiffusion. Strong precipitin lines were observed with the immunizing antigen and purified Le^a blood-group-specific glycoprotein[4] (Lloyd et al., 1968) but not with BSA or the Le^b– and Le^d–BSA conjugates. Electrophoresis on cellulose acetate membranes using barbitol buffer (pH 8.6) showed only a single band with the same mobility as purified rabbit IgG.

Le^b cross-reacting antibodies were not detectable by immunodiffusion. Nevertheless, since the Le^a and Le^b antigenic determinants share common structural features, the isolated anti-Le^a antibody solution was batched adsorbed with Le^b immunoadsorbent (50 mg/mL) for 1 h at 4 °C. The Le^b immunoadsorbent was removed by centrifugation and washed with phosphate-buffered saline (1 mL/200 mg). The combined supernatant and washings were filtered through a 0.2-μm filter and stored at 4 °C. Although approximately 10% of the antibody protein was lost in these manipulations, no change in activity was detectable by immunodiffusion.

(*b*) *Anti-Lewis b.* The procedure for the preparation of rabbit anti-Le^b antibody reagent was similar to the preparation of the anti-Le^a reagent, except that Le^b–BSA antigen was used for immunization. The antibodies were initially isolated from a six-rabbit pool (120 mL) of hyperimmune sera on a Le^b column and further purified by passing the solution through columns of the Le^a and Le^d immunoadsorbent. Nearly 80% of the antibody protein was removed by these adsorptions. The purified Le^b antibody solution showed reactions on immunodiffusion against both the Le^b–BSA conjugate and HLe^b blood-group specific glycoprotein[4] (Schiffman et al., 1964). No reactions were observed with BSA or the Le^a– or Le^d–BSA conjugates. Electrophoresis, as before, showed only a single band with the same mobility as rabbit IgG.

(*c*) *Anti-Lewis d.* The anti-Le^d reagent was prepared from antibodies isolated from a two-rabbit pool (40 mL) of hyperimmune serum raised to the Le^d–BSA conjugate. Affinity chromatography on a column bearing the Le^d hapten, in a manner similar to that described above for the isolation of the anti-Le^a and Le^b antibodies, provided a solution (2.6 mg/mL antibody protein) which, on examination by immunodiffusion, showed a strong precipitin line against the immunizing antigen and a very weak line against a βDGlcNAc–BSA conjugate. No precipitation was seen with BSA or the αLFuc(1→2)-βDGal–, Le^a–, Le^b– or αLFuc(1→2)βDGal(1→4)βDGlcNAc (H Type 2)–BSA conjugates. For removal of the detectable antibodies which cross-reacted with βDGlcNAc and possible antibodies having an affinity for Le^b, the antibody solution was batch adsorbed with βDGlcNAc and Le^b adsorbents (40 mg/mL). This procedure resulted in about a ~25% loss in antibody protein. The adsorbed antibody solution now showed a line of precipitation against only the immunizing antigen.

Preparation of the Tissue-Typing Reagents. For preparation of a given tissue-typing reagent, the stock solution of the purified antibodies was mixed with a solution of BSA in phosphate-buffered saline to achieve final concentrations of 0.5 mg/mL antibody protein and 10 mg/mL BSA. The addition of BSA minimized nonspecific adherence of antibody to the tissue (Schwarting & Marcus, 1979).

The Tissue Specimens. Patients who were scheduled for diagnostic upper gastrointestinal endoscopy had Lewis and ABH typing done by hemagglutination (Ortho). Direct-vision mucosal biopsies were taken from the duodenal bulb, distal antrum of the stomach, and the midbody of the stomach on the greater curvature. The biopsies were oriented, fixed in modified (1% acetic acid) Bouin's solution, and processed for paraffin-embedded serial sections as previously described (Perera et al., 1975). Biopsies with full-thickness mucosal inflammation or with gastric intestinalization were excluded. A total of 2 of 20 duodenal, 5 of 16 gastric antral, and 9 of 20 gastric body biopsies exhibited superficial inflammation. All the remaining biopsies were histologically normal. A total of 4 of 20 antral biopsies were of transitional gland type.

The Tissue-Staining Procedure. Unstained or hematoxylin- and eosin-stained sections were hydrated for the indirect immunofluorescence technique (Weinstein & Lechago, 1977). The antibody preparations (Lewis reagents) (0.5 mg/mL) were applied for 1 h. This was followed by a 15-min wash in phosphate-buffered saline, pH 7.2, and then a 1-h incubation with fluorescein-labeled goat–antirabbit IgG (ICN Pharmaceuticals, Inc.), diluted 1:10. After another wash in phosphate-buffered saline, the sections were dehydrated and coverslipped by using nonaqueous Uvinert. Experiments were done by applying each of the three Lewis reagents to three slides containing serial sections from a biopsy. In this way the results in adjacent sections could be examined and compared for the three Lewis reagents. Care was taken to keep the slides treated with a given Lewis reagent in separate phosphate-buffered saline wash containers in order to avoid cross-contamination.

Control incubations for each biopsy were done by substituting 1% BSA in phosphate-buffered saline for the Lewis reagent and also by omitting the first step (Lewis reagent) in the indirect immunofluorescence procedure.

Biopsy sections were examined by using the Leitz fluorescence microscope fitted with an Orthomat-W camera. Fluorescence intensity was graded on a scale of 0 (no fluorescence) to 4 (intense fluorescence). Grades 1 and 2 represent weak-staining intensities which were usually difficult to record on photomicrographs. Grades 3 and 4 signify strong staining. One other grading category was supranuclear (sn). This refers to a stippling of the cytoplasm in the supranuclear

[4] Blood-group specific glycoproteins (Le^a, N-1 20%, 2×; HLe^b, JS phenol insoluble) were generously donated by Professor E. A. Kabat, Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York.

Table I: Immunofluorescence Results in Gastric and Duodenal Mucosa for Lewis Antigens in H Blood-Group[a] Individuals

Lewis group[a] (secretor status):	Le^a (sese)			Le^b (Se)			Le^{a+b+} (Se)			Le^{a-b-} (Se)			Le^{a-b-} (sese)		
antiserum typing reagent:	Le^a	Le^b	Le^d	Le^a	Le^b	Le^d	Le^a	Le^b	Le^d	Le^a	Le^b	Le^d	Le^a	Le^b	Le^d
duodenum		—6[b]—			—9—			—1—			—3—			—1—	
brush border	3	2	0	0–1	4	2	4	4	0	0–1	2	4	1	0	1
enterocytes	sn–2	0–sn	0	sn	3	sn	sn	sn	0	0	sn	sn	0	0	2
goblet cells	3	1	0	2	3	3	4	2	0	0	0–2	4	0	1	0
Brunner's glands	1	1	0	1	1	0–1	2	1	0	1	2	0	2	1	0
gastric antrum		—4—			—8—			—1—			—2—			—1—	
surface cells	3	0–1	0	0	3	sn–2	2	1	0	0	0	3	0	0	1
neck cells	4	0–2	0–sn	0	3	sn–2	2	2	0	0	sn–2	3	2	sn	1
glands	1	2	0	2	1	0–1	1	1	0	1	sn–1	0	2	1	0
gastric body		—5—			—10—			—1—			—3—			—1—	
surface cells	3	0–1	0–sn	0	2	sn–2	3	1	0	0	0	2	0	0	1
neck cells	4	0–2	0–sn	0–2	4	sn–2	4	2	1	0	0–2	3	sn	0	2
glands	1	0–1	0	0–2	0–1	0	1	2	0	1	1	0	2	1	0

[a] As determined by hemagglutination. [b] Number of patients in each group. [c] Staining intensity was graded on a scale of 0 (no fluorescence) to 4 (intense fluorescence); sn refers to faint stippling in the supranuclear zone.

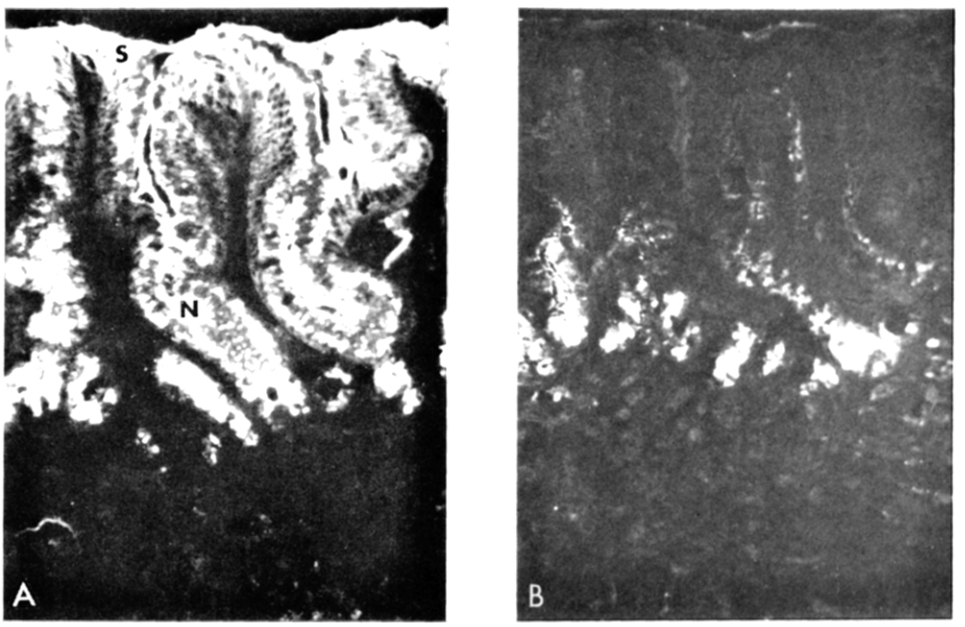

FIGURE 1: Indirect (double-layer) immunofluorescence in gastric tissue from an HLe^a Individual. (A) Gastric body section treated with the anti-Le^a reagent (112.5×). The surface (S) and neck (N) epithelial cells stain intensely. The underlying glands are negative (112.5×). (B) An adjacent section from the same biopsy as in (A), treated with the anti-Le^b (inappropriate) reagent (112.5×). The surface and upper neck cells are negative. There is positive staining of some of the deep neck cells (112.5×).

zone of cells. This sn pattern was distinct from the usual pattern of diffuse cytoplasmic fluorescence.

Biopsy sections were evaluated independently by C.M.S. and W.M.W. Averages are given for each structure that was assessed. Where there was a wider range of results or only a few patients in a group, then the range of results is given in Table I.

Results

Representative immunofluorescence staining patterns in tissue are shown in Figures 1 and 2. Background (nonspecific) fluorescence was negligible in tissue sections. The control sections were negative. The results are outlined in Table I.

Anti-Lewis-a Reagent. In Le^a individuals, the fluorescence was intense and diffusely distributed in the brush border and goblet cells of the duodenum and in the surface and neck cells of the stomach (Figure 1A). The glands of the duodenum and stomach stained much less brightly than the overlying epithelium. In Le^b patients, the Le^a reagent gave a negative or only faintly positive reaction in the brush border and an sn pattern in enterocytes of the duodenum and was negative in the surface cells of the stomach. In the Le^b patients, goblet-cell staining was focal at intensity 2, as was the staining in the glands of the gastric antrum and body. In the latter, some biopsies were negative. The same general pattern, i.e., weakly positive gland staining, was observed in patients who were Le^{a-b-}, either secretor or nonsecretor.

Anti-Lewis-b Reagent. In Le^b patients, fluorescence was intense in the brush border, the enterocytes and goblet cells of the duodenum, and the surface and neck cells of the stomach. In the glands, fluorescence was faint in the duodenum and negative to faint in the gastric antrum and gastric body. In the Le^{a+b+} patient, the Le^b staining was less intense than Le^a staining in goblet cells and gastric surface cells. In Le^a patients, there was focal positive staining in deep neck zones of the gastric body (Figure 1B). Many of the sections were completely negative. In Le^a individuals, there was faint gland fluorescence comparable in intensity to that found in the Le^b

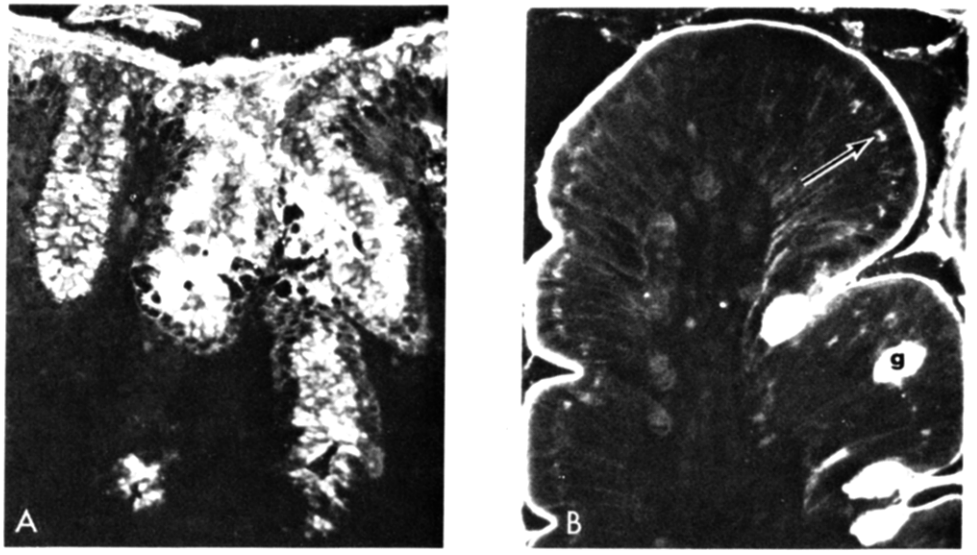

FIGURE 2: Indirect immunofluorescence with the anti-Le^d reagent. (A) Gastric body section from an HLe^{a-b-} secretor (180×). There is intense staining of surface and neck cells with anti-Le^d reagent. (B) Villus tip in a duodenal section from an HLe^{a-b-} secretor (375×). With the anti-Le^d reagent, there is intense staining of the brush border and goblet cells (g). Note also the faint supranuclear stippling (arrow).

patients. In the Le^{a-b-} individuals, secretor or nonsecretor, staining was negative or faint.

Anti-Lewis-d Reagent. In the Le^a patients, staining reactions were negative except for a focal faint sn pattern in gastric antral and body epithelial cells. In the Le^b individuals (excluding the Le^{a+b+} person), the brush border and most goblet cells were positive. There was a faint sn pattern in enterocytes and variable weak staining in Brunner's glands. In the gastric antrum of Le^b patients, the fluorescence ranged from sn to 2 in epithelial cells and was negative or faintly positive in the glands. The same pattern held for gastric body epithelial cells except that the glands were negative. In the Le^{a+b+} patient, all regions were negative except for a faint positivity in gastric body neck cells. In the Le^{a-b-} secretor patients, the Le^d staining reaction was striking. The epithelial cells of the gastric antrum and body (Figure 2A) were strongly positive. In the duodenum, the brush border and goblet cells also stained intensely (Figure 2B). In contrast, the staining pattern in the Le^{a-b-} nonsecretor was very faint.

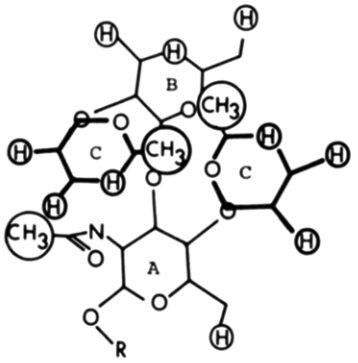

FIGURE 3: Projection of a molecular model for the Lewis-b determinant in order to indicate that the two αLFuc residues reside in close proximity and near touching above the β,β′ side of the βDGal(1→3)βDGlcNAc disaccharide unit. H = hydroxyl, A = βDGlcNAc, B = βDGal, C = αLFuc.

Discussion

The discovery of the Lewis blood group is usually attributed to Mourant (Mourant, 1946) who, in 1946, described the new antibody subsequently termed anti-Le^a in the sera of two post partum women whose babies suffered from hemolytic disease of the newborn. It is now apparent that this observation was anticipated by Ueyama (Ueyama, 1939; Furuhata & Ueyama, 1939), who detected the Le^a antigen in the saliva of nonsecretors. The second member of the Lewis family, anti-Lewis-b serum, was found by Andresen (1948). When antisera with these two activities was used, it was found that erythrocytes could be classified as one of four phenotypes, Le^a, Le^b, Le^{a-b-}, or Le^{a+b+} (extremely rare).

Studies using animal sera raised to human salivary substances (Iseki et al., 1957; Potapov, 1970, 1976) have shown that it is possible to serologically classify Le^{a-b-} individuals into two phenotypes which were tentatively described as Le^c and Le^d.

The carbohydrate nature of the Le^a and Le^b antigenic determinants of blood-group-specific glycoproteins obtained from ovarian cysts was deduced by Watkins & Morgan (1957) from inhibition studies involving oligosaccharides derived from human milk. Subsequently, degradation of Le^a- (Rege et al., 1964) and Le^b- (Marr et al., 1967) specific glycoproteins allowed the assignment of the currently accepted structures for these determinants. The structure of the Le^a determinant has been confirmed both by the elucidation of the structure of a Le^a-specific glycosphingolipid by Smith and co-workers (Smith et al., 1975) and by chemical synthesis (Lemieux et al., 1975). Recently, Graham and co-workers (Graham et al., 1976, 1978) established the structure for the Le^d determinant.

To the best of our knowledge, the reagents reported herein mark the first time that antisera raised against artificial antigens bearing totally synthetic oligosaccharide haptenic groups have been used to detect the Lewis antigens in human tissue. Antisera with Lewis activity have previously been prepared by immunization of animals with either natural substances (Marcus & Grollman, 1966; Iseki et al., 1957; Potapov, 1976) or artificial antigens bearing haptenic groups isolated from natural sources (Zopf & Ginsberg, 1975).

A particular advantage of the ability to synthesize the desired determinant and subcomponent structures is that immunoadsorbents can be prepared both for the isolation of the desired activity and also for the removal of unwanted cross-reacting antibodies. This is best illustrated in the case of preparation of the anti-Le^b reagent.

The conformation of the Le^b determinant can be approximately depicted by the projection formula in Figure 3 (Lemieux et al., 1980a). It was found that the orientations of the

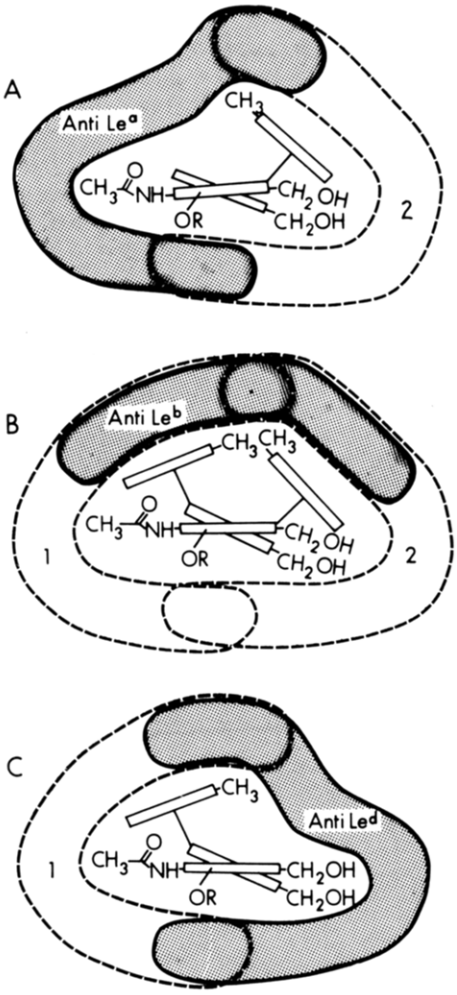

FIGURE 4: Schematic representations of the structures for the Le^a(A), Le^b(B), and Le^d(C) determinants in order to illustrate the topographical regions about the Le^b determinant which are common to the Le^a and Le^d structures and, thereby, to suggest that antibodies which are specific for the Le^b determinants likely bind regions which involve both the fucose units.

αLFuc groups are maintained in the precursor Le^a and Le^d determinants. Thus, the Le^a and Le^d determinants have topographical features in common with the Le^b determinant, and this is demonstrated schematically in Figure 4. The purpose of these diagrams is to show that region 2 or the Le^a determinant (see Figure 4A) is present as region 2 of the Le^b determinant (see Figure 4B) and that region 1 of the Le^d determinant (Figure 4C) is common to region 1 of the Le^b determinant. Thus, the heterogeneous population of antibodies raised against the Le^b artificial antigen was expected to contain antibodies that would recognize and combine with the other two Lewis determinants. In fact, it was found that the antibodies raised against the Le^b artificial antigen and isolated by affinity chromatography with the Le^b adsorbent showed strong cross-reaction with both the Le^a and Le^d determinants as assessed by immunodiffusion. Indeed, passage of the crude anti-Le^b antibody solution through columns of Le^d and Le^a immunoadsorbents removed nearly 80% of the antibodies. The antibodies that remained in the eluate then showed no immunodiffusion precipitin lines against either BSA or the Le^d or Le^a artificial antigens but provided a strong reaction against the Le^b artificial antigen as well as against HLe^b human blood-group-specific glycoprotein. On the basis of the schematic representation for the Lewis antigens in Figure 4, these anti-Le^b-specific antibodies may be expected to have combining sites spreading over both the αLFuc residues, as is indicated by the shaded portion of Figure 4B.

For the Le^a and Le^d antibodies, the cross-reactivity with inappropriate Lewis antigens was anticipated to be less pronounced as these smaller antigenic determinants have fewer common structural features. For the Le^a-antibody preparation, no anti-Le^b activity could be detected by immunodiffusion. Nevertheless, the initially isolated antibodies were refined by adsorption with the Le^b immunoadsorbent. This treatment removed about 10% of the antibodies. The final preparation gave no precipitin line against HLe^b human blood group specific glycoprotein[4] but did give a precipitin line with a Le^a blood-group substance. The crude anti-Le^d reagent showed no detectable cross-reaction with BSA or either Le^a or Le^b conjugates. Since a weak line of precipitation was observed against a (βDGlcNAcO$(CH_2)_8$CONH)$_{18}$–BSA conjugate, the antibody solution was adsorbed with the corresponding immunoadsorbent. Adsorption with the Le^b immunoadsorbent was also performed as a precautionary measure. These refined anti-Le^a and anti-Le^d reagents, because of the adsorptions with the Le^b immunoadsorbents, are expected to have antibodies directed against their determinants mainly in the shaded regions of Figure 4 (A and C, respectively). It is of interest to note that the anti-Le^a antibody population, on this basis, would bind the regions about the acetamido group of the Le^a determinant.

The antibody reagents when used in the indirect immunofluorescence procedures produced crisp fluorescence staining patterns with negligible background staining. It was also found that the reagents could be used in studies of paraffin-embedded tissues that have been processed in a conventional fashion. Cryostat sections are not required (Glynn et al., 1957; Szulman & Marcus, 1973). This permits retrieval of stored sections from tissue files for study. We did not do a formal comparison with formalin-fixed samples, but, in some prelinary studies, we found that these samples gave suitable results. The anti-Le^b reagent reported herein has also been used to demonstrate the Le^b antigen in resin-embedded sections by using the immunoperoxidase method (Heyderman & Monaghan, 1979). The results obtained with these reagents in the course of the examination of tissues from H blood-group patients will now be discussed.

Intense fluorescence (grades 3 and 4) was expected to identify the determinant for which the antibody reagent was designed. In fact, examination of the tissues stained with the anti-Lewis reagents made it possible to easily predict the Lewis phenotype of an individual. The Lewis-a, -b, and -d antigens were most concentrated in the lining cells of the stomach and duodenum. On the other hand, goblet cells and Brunner's gland of the duodenum and the deep glands of the stomach often stained faintly with the anti-Le^a and anti-Le^b reagents irrespective of Lewis phenotype. This phenomenon was generally not observed with the anti-Le^d reagent. It is important to consider what negative or weak staining may represent.

First of all, a negative staining result using an immunohistochemical reagent does not necessarily indicate total absence of the antigen for which the reagent was designed. Instead, the result may simply indicate that, under the given set of experimental conditions, a relatively lesser amount of the determinant was not detected. These conditions include such factors as fixation methods and length of the incubation time. Alternatively, appearance of very weak staining intensities (grades 1 and 2, Table I) does not necessarily require that the reagent is locating and identifying the presence of the determinant for which the reagent was designed. Thus, for example, the weak-staining reactions observed (see Table I

and Figure 1B) by using the anti-Le^b reagent on Le^a tissues does not necessarily mean that these tissues have some abundance of Le^b determinants. At present, these low-intensity stains are expected to arise either from Le^b or Le^a determinants or from some other as yet unidentified structure which, under the experimental conditions used, showed measurable affinity for the anti-Le^b antibodies. For example, the Le^b determinant and the H (type 2) related structure, αLFuc(1→2)βDGal-(1→4)[αLFuc(1→3)]βDGlcNAc, which has been termed the Y determinant (Kobata, 1975), can be expected to have substantial portions of their structures nearly identical, and some cross-reactions would not be surprising. Consequently, it is considered important to examine the nature of these weak-staining reactions, and we plan to do this.

The anti-Le^d [anti-H type 1] antibodies which were used in these histochemical studies were not precipitated (immunodiffusion) by the artificial antigen prepared from H (type 2) hapten (R.U. Lemieux and O. Hindsgaul, unpublished results). Therefore, even though the presence of Le^d determinants (H type 1) could not be demonstrated in the HLe^a individuals (Table I), the H (type 2) determinants could have been present in the tissues which were examined. Indeed, previous studies using anti-H reagents of animal (Glynn et al., 1957), human (Szulman, 1962), and plant (Kent, 1964) origin have unequivocally demonstrated the presence of H antigens in the gastric tissues of nonsecretor HLe^a individuals. Therefore, these individuals undoubtedly possess the αLFuc transferase necessary to synthesize the H (type 2) determinant. Why these individuals are not also Le^d [H type 1] positive is then an interesting question.

On the basis of recent studies, it appears that the Le^b determinant can arise by action of the appropriate αLFuc transferase on either the Le^a or the Le^d determinant (Prohaska et al., 1978; Schenkel-Brunner & Prohaska, 1978; Shen et al., 1968). However, the process βDGal(1→3)βDGlcN(Ac) (Le^c?) → Le^d → Le^b appears to be most efficient and likely the important route. Indeed, the inability of Le^b individuals to form anti-Le^a is attributed to the presence of Le^a antigens (Judd et al., 1978); i.e., for Le^b individuals, Le^a is a self-antigen. As can be seen from the data presented in Table I, Le^b individuals appear to show the presence of all three Lewis antigens; however, by far the strong reaction was with the appropriate antibody preparation. For these reasons, it is always possible that the biosynthesis of the Le^b determinant from the immediate precursor was not quantitative, and, therefore, the tissue of a given Le^b individual would show the presence of one or both of the precursor Le^d and Le^a antigens as well as the Le^b antigen.

In view of the biosynthetic origins of the Lewis antigens, it was to be expected that tissues derived from Le^{a-b-} people would fall into one of two groups: (1) those whose tissues would show the presence of the Le^d antigens but neither Le^a nor Le^b antigens and (2) those in whose tissues neither Le^a, Le^b, nor Le^d antigens would be present. As seen from Table I, the tissues from the three Le^{a-b-} *secretors* showed strong positivity only with the Le^d reagent and thus appear to belong to the first group described above. It is possible that the single Le^{a-b-} *nonsecretor* was a member of the second group since the tissue of this individual did not show a marked positivity with any of the Lewis reagents. Firm conclusions cannot be based on the results obtained from one individual, but these results indicate that the Le^d determinant is the key marker for ABH secretor status.

As a result of this investigation, it became apparent that the process which leads to the formation of the Le^d determinant is central to ABH secretor status and that this process is not expressed in HLe^a individuals. Furthermore, it became apparent that the Le^a determinant is present in the N-acetylated form. It was pointed out (Lemieux, 1978) that the N-acetylated form of the type 1 disaccharide is not likely (for stereochemical reasons) a substrate for the αLFuc transferase that converts the type 2 disaccharide to the H (type 2) determinant. Thus, the question arose as to whether or not the Le^d and Le^b antigens which occur in the saliva of secretors are present in the free amine forms rather than in the usually accepted *N*-acetyl forms (Lemieux et al., 1980b).

The structures of the Lewis antigenic determinants require that these arise by way of fucosylation of a form of the type 1 disaccharide, namely, 2-amino-2-deoxy-(3-*O*-β-D-galacto-pyranosyl)-β-D-glucopyranose, as the terminal unit of a precursor oligosaccharide structural unit of either a glycoprotein or glycolipid blood-group-active substance. Whether or not these antigens have these determinants in the N-acetylated form is not thoroughly established. The N-acetylated form appears definitely to be the case for the Le^a-active glycosphingolipid which was isolated and unambiguously characterized (Smith et al., 1975). This also applies to the H (type 2)-active glycosphingolipid which was characterized by Hakomori and co-workers (Stellner et al., 1973). However, the methodologies used for the elucidation of the structures of the Lewis-group-specific glycoproteins obscure this consideration (Marr et al., 1967; Rovis et al., 1973). Furthermore, these substances were collected from ovarian cysts and could prove to be structural anomalies, such as the occurrence of both the Le^a- and Le^b-glycolipids in human adenocarcinoma tissue, regardless of the Lewis blood type of the donor of the tumor (Hakomori & Andrews, 1970).

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Photochemistry and Fluorescence of Bacteriorhodopsin Excited in Its 280-nm Absorption Band†

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ABSTRACT: Photochemical and fluorescence studies are carried out, exciting bacteriorhodopsin (BR) in its 280-nm absorption band. The data indicate that energy transfer takes place, with a quantum yield of 0.7–0.8, from excited tyrosines and tryptophans to the retinyl chromophore. All of the tyrosine and five to six tryptophan residues are completely quenched by the transfer process while one tryptophan is unquenched and one is partially (~80%) quenched. Energy transfer to the chromophore leads to a photocycle identical with that triggered in (light adapted) bacteriorhodopsin by excitation within the visible absorption bands of the chromophore. The emissive properties of BR in the intact membrane are found equal to those of a Triton X-100 solubilized BR monomer. The energy transfer data are discussed in terms of the available amino acid sequence and the electron density map of bacteriorhodopsin. Although such data cannot suggest a single fit between the sequence and the density map (one out of the 7! = 5040 possibilities), they do provide a criterion for testing any specific model for the structure of bacteriorhodopsin.

Bacteriorhodopsin, the protein pigment of the purple membrane of *Halobacterium halobium*, is responsible for a light-driven proton pump which leads to ATP synthesis. [For a review, see Stoeckenius et al. (1978).] Analogous to visual pigments [for a review, see Ottolenghi (1980)], the spectrum of bacteriorhodopsin in the visible and in the near-UV is characterized by three absorption bands (Becher et al., 1978). The α band (with an extinction of $\epsilon \approx 63\,000$ M^{-1} cm^{-1}) peaks, in light-adapted (all trans) bacteriorhodopsin (BR^{l}_{570}), at 570 nm. A lower extinction band ($\epsilon \approx 13\,000$ M^{-1} cm^{-1}), lacking a clear maximum, covers the range between 450 and 300 nm (β band). In the UV spectrum, an intense ($\epsilon \approx 75\,000$ M^{-1} cm^{-1}) band is observed, peaking at 280 nm (γ band). Both α and β bands are exclusively due to the retinyl chromophore, which is attached to the protein via a protonated Schiff-base linkage. In keeping with the characteristic protein fluorescence induced by excitation in the near-UV spectrum (Bogomolni

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