Topography and Polypeptide Distribution of Terminal N-Acetylglucosamine Residues on the Surfaces of Intact Lymphocytes

EVIDENCE FOR O-LINKED GlcNAc*

Carmen-Rosa Torres‡ and Gerald W. Hart§

From the Department of Physiological Chemistry, The Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205

Bovine milk galactosyltransferase has been used, in conjunction with UDP-[3H]galactose, as an impermeant probe for accessible GlcNAc residues on the surfaces of lymphocytes. Galactosylation of living thymic lymphocytes is dependent upon cell number, enzyme concentration, UDP-galactose concentration, and Mn** concentration. Kinetics of labeling are biphasic, leveling off at approximately 30 min. The data strongly indicate vectorial surface labeling and covalent attachment of galactose. Thymocytes, T-lymphocytes, and B-lymphocytes have approximately 10⁶, 3 × 10⁶, and 5 × 10⁶ galactosylatable sites on their cell surfaces, respectively. Numerous proteins are exogalactosylated that differ quantitatively among the major functional subsets of lymphocytes. Negligible radioactivity is found in lipid. In thymocytes, 49% of the exogalactosylated oligosaccharides are alkali labile, whereas 80 and 90% of that derived from T-lymphocytes and B-lymphocytes can be β-eliminated, respectively. Sensitivity of the intact proteins or tryptic peptides to the peptide:N-glycosidase also confirms the relative amounts of cell surface, N-linked and O-linked oligosaccharides which are exogalactosylated. Composition, size, and high performance liquid chromatography on two types of high resolution columns establish that the bulk of the exogalactosylated, β-eliminated oligosaccharides are Galβ1-4GlcNAcR. These data suggest the presence of O-glycosidically linked GlcNAc monosaccharide on many lymphocyte cell-surface proteins. However, additional experiments indicate that the majority of these moieties appear to be cryptic or inside the cell. Thus, these studies not only describe dramatic differences in the amounts and distribution of terminal GlcNAc residues on phenotypically different lymphocyte populations, but they also describe the presence of a novel protein-saccharide linkage, which is present on numerous lymphocyte proteins.

Nearly all of the characterized lymphocyte differentiation or histocompatibility antigens are cell-surface glycoproteins (1). In fact, the expression of specific membrane glycoproteins has proven to be the most reliable marker for functionally distinct subsets of lymphocytes. Functional subsets of lymphocytes can readily be separated using monoclonal or polyclonal antibodies to these specific membrane glycoproteins (2). Interestingly, similar functional subsets of lymphocytes can also be isolated solely based upon their specific reactivity to glycosyl-binding plant lectins (3–5).

The biological functions of lymphocytes are mediated by a complex network of cellular interactions in which these cell-surface glycoproteins are directly involved (6, 7). Recent work suggests that the saccharide moieties on specific lymphocyte glycoproteins play an essential role in their biochemical functions (8, 9). Several studies have demonstrated pronounced changes in the saccharide topography or in the glycosylation of particular proteins on the surfaces of lymphocytes accompanying lymphocyte activation or development (10–21). Most of these earlier studies have involved the use of lectin binding (10, 15), galactose oxidase/sodium borotritide surface labeling (13, 21), whole cell electrophoresis (16, 20), or gel electrophoresis (17, 19). An understanding of the changes in oligosaccharide structural diversity accompanying lymphocyte activation and development represents a first step toward ascertaining the details of the structural-functional significance of the varied glycosylation of these specific lymphocyte cell-surface receptors.

Only recently has it become feasible to use purified glycosyltransferases as specific vectorial modifiers of saccharide structure and as probes for exposed saccharide moieties on intact cells (22). This is largely due to the development of techniques, based upon affinity chromatography, for the purification of many of these enzymes (22–27). Glycosyltransferases are remarkably specific for both their acceptor substrates and their donor sugar nucleotide. In fact, there may be a specific glycosyltransferase for the synthesis of every type of glycosidic linkage found in nature (28). Recent work has directly demonstrated the practical use of purified glycosyltransferases as vectorial cell-surface probes and as modifiers of oligosaccharide structures (22, 29–31).

Bovine milk galactosyltransferase (EC 2.4.1.38), in the absence of β-lactalbumin (32), catalyzes the following reaction, where R can be any of several different moieties.

\[
\text{UDP-galactose} + \text{GlcNAc-R} \rightarrow \text{Galβ1-4GlcNAc-R} + \text{UDP}
\]

It is the most extensively studied of any mammalian glycosyltransferase (25–27, 32). As part of our long term investigations into the roles of cell-surface saccharide moieties in the development and functions of lymphocytes, we have selected this highly purified bovine galactosyltransferase to test the feasibility of using glycosyltransferases to map "the" cell-surface topography of lymphocytes as a function of their phenotype, metabolic state, and developmental stage. In these

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initial studies, we have demonstrated pronounced differences in the disposition of terminal GlcNAc residues on the surfaces of lymphocyte subpopulations having functionally different phenotypes. Additionally, the data strongly indicate that the bulk of their terminal GlcNAc residues, which are exogalactosylated, originally existed as single monosaccharide units O-glycosidically linked to protein.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Conditions for the Exogalactosylation of Lymphocytes**—The conditions for the use of highly purified bovine milk galactosyltransferase as an impermeant probe of lymphocyte cell-surface saccharide topography were established. Fig. 1 summarizes the results of experiments which determined the optimal conditions for galactosylation of living lymphocytes (90–99% viable by Trypan blue exclusion). The labeling of lymphocyte surface proteins was shown to be dependent upon UDP-galactose, cell concentration, and Mn2+ ion concentration. Likewise, the reaction was dependent upon galactosyltransferase concentration, and the kinetics of labeling were biphasic, leveling off at about 30 min. These biphasic kinetics, as shown in Fig. 1D, were very reproducible from three totally independent experiments, suggesting that about 60% of the galactosylatable proteins of thymocytes were labeled more rapidly than the remaining 40% of the surface acceptors. However, in these initial studies, we chose conditions for saturation labeling of galactosylatable surface components in order to assess the topography of terminal GlcNAc residues on the surfaces of lymphocytes which have different functional phenotypes.

Prior autogalactosylation of the transferase (47), and the presence or absence of heat-inactivated serum in the labeling buffer had no effect on either the amount of incorporation, the SDS-PAGE profiles, or pronase glycopeptide profiles of the labeled lymphocyte components (data not shown). In addition, the levels of [3H]galactose incorporated into galactosyltransferase itself were insignificant compared to that incorporated into lymphocyte glycoproteins. Identical levels of incorporated radioactivity and SDS-PAGE or pronase glycopeptide profiles were obtained with both α-lactalbumin affinity purified galactosyltransferase and with the commercially available enzyme (data not shown).

**Evidence for Vectorial Surface Labeling**—Results of several experiments strongly indicate that virtually all of the radioactivity incorporated into lymphocytes is the result of vectorial galactosylation at the cell surface. 1) Addition of a 1000-fold excess of unlabeled galactose, as in 1) above. 3) In vectorially labeled cells, virtually 100% of the incorporated radioactivity was still in the form of galactose, indicating that no epimerization had occurred. 4) Examination of total cellular associated radioactivity after standard labeling conditions indicated that generally greater than 96% was acid insoluble when cells were vectorially labeled with UDP-[3H]galactose and galactosyltransferase, whereas less than 5% of the cell-associated radioactivity was acid insoluble when an equivalent amount of [3H]galactose was used. 5) Addition of 0.1 mM galactose 1-phosphate or 2 mM UDP-GlcNAc had little or no effect on incorporation of radioactivity by exogalactosylation (control, 6.3 × 105 cpm/106 cells; galactose 1-phosphate, 6.3 × 105 cpm/106 cells; UDP-GlcNAc, 4.5 × 105 cpm/106 cells). 6) Under standard incubation conditions, greater than 80% of the added UDP-[3H]galactose remained intact and inhibited the hydrolysis by 20% by the addition of 5'-AMP (2.5 mM) had no effect on levels of radioactivity incorporated. 7) Cell viability was typically greater than 95% after the galactosylation reaction. 8) Autoradiography of labeled lymphocytes (greater than 95% viable) indicated that at least 36% of the cells were heavily labeled. This percentage of labeled cells is a minimal estimate, since in the technique employed only the coverslip contained photographic emulsion (48). All of the radioactivity observed in these autoradiographic analyses of exogalactosylated lymphocytes was localized in massive “caps” at the poles of the cells rather than being evenly distributed along the periphery (manuscript in preparation). These data clearly indicate that our labeling results are not due to the selective galactosylation of small numbers of lysed or dead cells. However, it is likely that exogalactosylation is preferentially labeling certain subclasses or developmental stages of lymphocytes (see below).

**Vectorial Galactosylation by Endogenous Versus Exogenously Added Galactosyltransferase**—The levels of galactose incorporated from UDP-[3H]galactose into membrane proteins of thymocytes were at least 10–20 times higher in the presence of exogenously added galactosyltransferase (Fig. 1C).

**Nature of the Membrane Associations of Vectorially Galactosylated Proteins**—Exogalactosylated thymocytes were subjected to different extraction procedures to evaluate whether the galactosylated surface proteins were integral or more peripheral membrane components (50). Secreted or very loosely associated proteins, which could be washed off by isotonic labeling media, were not examined in these studies. Nearly all of the cell-associated radioactivity was on proteins, since chloroform:methanol (2:1) extraction removed only 3%. Only 4% of the cell-associated, galactosylated products were extractable by either hypotonic buffer or by 5 mM EDTA (under conditions of cell lysis). When labeled thymocytes were extracted with NP-40, 34% of the radioactivity was initially solubilized with 0.05% detergent and consecutive extraction with 0.1% NP-40 increased the solubilized radioactivity by only 9%. Virtually all of the radioactivity was solubilized by SDS-PAGE sample buffer. Taken together, these data indicate that the bulk of the cell-associated lymphocyte surface proteins, which are externally galactosylatable, are integral membrane proteins.

**Differential Exogalactosylation of B-, T-, and Thymic Lym-
phocytes—Purified B-, T-, and thymic lymphocytes were isolated and assessed for purity by fluorescent antibodies. Thymocyte preparations were routinely greater than 99% lymphocytes by microscopic examination (33). T-lymphocytes were typically 95–98% pure, and B-lymphocytes were generally 95–100% pure. The viability of these purified cell populations, in these particular experiments, ranged from 98–99, 95–98, and 53–90% for thymocytes, T-lymphocytes, and B-lymphocytes, respectively. The enzyme dependence for the exogalactosylation of purified populations of these lymphocyte subsets is shown in Fig. 2. Nearly identical results are obtained whether the data is normalized to protein or to cell number. Data from six different experiments indicate that T-lymphocytes show an average of at least 3× (range, 2.8-3.9×) more galactosylation than do thymocytes from the same mice. Similarly, data from five independent experiments show that B-lymphocytes have an average of at least 5× (range, 4.9-5.8×) more galactosylatable cell-surface sites than thymocytes.

The SDS-PAGE analysis of 3H-galactosylated proteins (Fig. 3) indicates that numerous proteins are labeled in all three cell types. In each case, the major labeled protein has a molecular weight of 76–78,000. In addition to numerous minor components, thymocytes contain three major protein bands which are labeled by external galactosylation of living cells (M, = 125,000, 105,000, and 76,000, respectively). All of these bands are also detectable in T- and B-lymphocytes. T-lymphocytes show four species in the 140K-105K range, and six bands at 78K, 69K, 50K, 26K, 24K, and 19K. Three of these bands (69K, 50K, and 19K) are not detectable in thymocytes, even with prolonged autoradiographic exposures. B-lymphocytes contain more labeled proteins of higher molecular weight, having six such bands, two of which (122K and 84K) are not detectable in either T-lymphocytes or in thymocytes from the same mice. Major quantitative differences among thymocytes and the splenic-derived lymphocytes are also seen in the proteins banding in the 30,000–50,000 molecular weight range. Band by band comparisons of these gels also shows numerous other quantitative differences in the labeling of specific size classes of proteins between the three types of lymphocytes (Fig. 3). The complexity of these labeling patterns and those obtained with lactoperoxidase cell-surface iodination or metabolic labeling with [35S]methionine has thus far precluded our determining whether these lymphocyte class-specific galactosylation patterns are the result of differences at the polypeptide level, glycosylation differences, or a combination of both. In any case, there appears to be profound quantitative and perhaps even some qualitative differences in the numbers and locations of cell-surface, terminal GlcNAc residues on functionally different subsets of lymphocytes.

**Demonstration That Vectorially Added [3H]Galactose Is Covalently Attached to Protein**—Several experiments were undertaken to ensure that the radioactivity was, in fact, covalently attached to protein. Evidence that the disaccharide results from galactosylation of a covalently attached moiety is summarized below. 1) Virtually all of the radioactivity on intact, detergent-solubilized proteins migrates in the void volume of Sephadex G-50, even in the presence of 4 M guanidine HCl. 2) Elimination of material excluded from Sephadex G-50 in the presence of 4 M guanidine HCl, followed by Bio-Gel P-4 chromatography of the reaction mixtures, yields nearly identical profiles as that shown in Fig. 6. 3) DPC-trypsin treatment of total reduced and alkylated, [3H]galactosylated proteins yielded a very complex profile of tryptic peptides on reverse phase HPLC (C-18), which still contained nearly all of the initial protein-associated [3H]galactose, especially when normalized for typical recoveries from these HPLC columns (41, 53). 4) Nearly all of the protein-associated [3H]galactose was recovered as limit pronase glycopeptides when exogalactosylated lymphocytes were exhaustively digested with pronase (Fig. 4). 5) Virtually all of the [3H]galactose on exogalactosylated lymphocytes was acid insoluble and migrated as typical protein bands upon SDS-PAGE.

**Nature of the Galactosylated Oligosaccharides on Lymphocyte Cell Surfaces**—In initial experiments, purified lymphocytes were exogalactosylated, and the acid-soluble cellular components were subjected to exhaustive digestion with predigested pronase (40) in order to study the size classes of labeled glycopeptides. As shown in Fig. 4, A and C, high resolution Bio-Gel P-4 (~400 mesh) chromatography indicates that most of the incorporated radioactivity in B-lymphocytes is found in four to six major limit pronase glycopeptide size classes, all of which are relatively low in molecular weight. On the other hand, thymocytes and T-lymphocytes yielded a higher proportion of larger molecular weight pronase glycopeptides (Fig. 4B). Repeated pronase digestion or even exhaustive sequential digestion of these glycopeptides with Aminopeptidase M (51) and carboxypeptidase Y (52) failed to substantially alter the gel-filtration patterns of the lower molecular weight glycopeptides (Fig. 4D). In spite of their resistance to proteolysis, these glycopeptides, in fact, contain considerable peptide heterogeneity when examined by high resolution reverse phase HPLC under conditions relatively insensitive to the nature or size of the attached oligosaccharide (41, 53). It seems likely that the resistance to complete proteolysis is conferred by glycosylation.

Labeled proteins from all three cell types were analyzed for the nature of the protein-saccharide linkage containing the [3H]galactose by treatment with dilute alkali in the presence of excess NaBH₄ (44). Fig. 5 (miniprint) shows the time course of this alkali catalyzed β-elimination of the [3H]galactosylated, intact proteins of splenic lymphocytes (mixture of T- and B-lymphocytes). Surprisingly, a large proportion of the radioactivity very rapidly becomes β-eliminated under these con-
Terminal GlcNAc on Lymphocyte Cell Surfaces

FIG. 3. SDS-polyacrylamide gel electrophoresis of exogalactosylated cell-surface proteins from functionally distinct types of lymphocytes. Living, purified lymphocytes were exogalactosylated and total acid-insoluble proteins were analyzed by SDS-PAGE (see text for details). Equivalent amounts of protein were loaded in each lane. A, 15 h of autoradiographic exposure; B, 66 h of autoradiographic exposure. 1, thymocytes; 2, splenic T-lymphocytes; 3, splenic B-lymphocytes. Standards (STD) are [14C]IgG (murine; heavy chain, Mr = 50,000; light chain, Mr = 25,000). Results from two independent experiments are shown.

FIG. 6. Bio-Gel P-4 chromatography of β-eliminated oligosaccharides from exogalactosylated cell-surface proteins of purified lymphocyte subsets. Living lymphocytes were purified, exogalactosylated, and labeled. Acid-insoluble proteins were β-eliminated as described under "Experimental Procedures." The entire reaction mixtures were then acidified, neutralized, and chromatographed on columns of Bio-Gel P-4 (1 × 195 cm; ~400 mesh). Arrows, elution positions of standards: V₀, [3H]mannose; 1, (GlcNAc); 2, (GlcNAc); 3, (GlcNAc); 4, (GlcNAc); Vₑ, cytochrome c. Samples from equivalent numbers of cells were analyzed (note the differences in scales).
these results and those involving dilute alkali to determine oligosaccharide-protein linkage is quite striking. Also, when trypic glycopeptides of exogalactosylated proteins from T-lymphocytes or B-lymphocytes were treated with the peptide:N-glycosidase and chromatographed on high resolution reverse phase HPLC (41, 53), there was almost no detectable difference in the complex trypic patterns as compared with untreated trypic glycopeptides, indicating that very little of the radioactivity was released as free oligosaccharide (data not shown). Since the peptide:N-glycosidase quantitatively releases N-linked saccharides from every one of the several lymphocyte N-linked glycoproteins which we have examined (41, 53), these results strongly suggest that the added galactose is not part of an unusual glycosic linkage involving terminal GlcNAc in N-linked oligosaccharides. It should be noted that peptide:N-glycosidase will also cleave a single unsubstituted GlcNAc-asparaginyl linkage in a glycopeptide, such as that left by the action of endoglycosidase H (42). Taken together, the β-elimination and glycosidase data clearly indicate that a greater proportion of N-linked oligosaccharides are exogalactosylated in thymocytes than in either of the two peripheral lymphocyte types. Furthermore, the results indicate that the bulk of the [3H]galactose incorporated into proteins by exogenous galactosylation is O-glycosidically linked and found on a disaccharide the size of Galβ1-4GlcNAcitol after β-elimination under reducing conditions.

Identification of the β-Eliminated, Exogalactosylated Disaccharide—in order to identify the [3H]galactosylated disaccharide, the peaks from β-eliminated samples, which had been chromatographed over Bio-Gel P-4, were pooled, lyophilized, and analyzed by HPLC on a styrene-based, sulfonic acid column (0.5 × 100 cm) in the calcium form (Fig. 9). These columns are capable of extremely high resolution of low molecular weight, neutral oligosaccharides. As shown in Fig. 9A, authentic disaccharides, Galβ1-4GlcNAcitol, Galβ1-

4GlcNAcitol, and Galβ1-3GlcNAcitol, which differ only in the position of a single hydroxyl moiety or linkage, are well resolved by this type of chromatography. The disaccharide obtained from β-elimination of exogalactosylated lymphocytes (all three cell types) exactly coelutes with the Galβ1-4GlcNAcitol standard, both by comparison of radiolabeled standards in separate runs and by co-chromatography of the standard and the unknowns, using ultraviolet absorbance (210 nm) to detect the standard disaccharide. HPLC analyses of the disaccharides on an amino-bonded column, which separates neutral saccharides by hydrogen-bonding interactions, also demonstrates that the unknown disaccharides from all three lymphocyte types exactly coelutes with authentic Galβ1-

4GlcNAcitol (data not shown). Treatment of the labeled proteins or oligosaccharides with highly purified β-galactosidase (Escherichia coli) indicated that most of the added galactose was terminal and of the correct anomeric configuration (69% released).

Exogalactosylation of Metabolically Radiolabeled Saccharides—in order to determine the relative amounts of the...
Terminal GlcNAc on Lymphocyte Cell Surfaces

Fig. 9. HPLC analysis of β-eliminated disaccharide from exogalactosylated lymphocyte cell-surface proteins. Purified lymphocyte subsets were exogalactosylated, labeled proteins were β-eliminated, and the major disaccharide products were isolated by gel-permeation chromatography on Bio-Gel P-4 (see "Experimental Procedures" for details). Isolated disaccharide alcohols were deionized by passage through a mixed-bed ion exchange resin and chromatographed on Bio-Gel P-4 (see "Experimental Procedures" for details). Isolated disaccharide alcohols were deionized by passage through a mixed-bed ion exchange resin and chromatographed on Bio-Gel P-4 (see "Experimental Procedures" for details). Isolated disaccharide alcohols were deionized by passage through a mixed-bed ion exchange resin and chromatographed on Bio-Gel P-4 (see "Experimental Procedures" for details). Isolated disaccharide alcohols were deionized by passage through a mixed-bed ion exchange resin and chromatographed on Bio-Gel P-4 (see "Experimental Procedures" for details).

DISCUSSION

The aim of this study was to establish the conditions for using bovine milk galactosyltransferase as an impermeant probe for terminal GlcNAc residues on the surfaces of various types of lymphocyte subpopulations. Our plan was to not only quantify and identify proteins containing these residues, but also to characterize the nature of the oligosaccharides to which they are attached. Previous studies have shown that bovine milk galactosyltransferase as a probe for terminal GlcNAc on isolated saccharides, microsomal vesicles, or cell surfaces (54-57). Analysis of galactosylated cell-surface glycoproteins, in many of the earlier studies on cell-surfaces, was performed by SDS-PAGE, isoelectric focusing, or a combination of both. However, the nature of the cell-surface exogalactosylated oligosaccharides has not been previously examined for any cell type.

Results described in this paper indicate the following: 1) Galactosyltransferase can readily be used to probe the accessibility of terminal GlcNAc residues on the surfaces of living lymphocytes, under conditions where virtually all of the incorporated [14C]galactose is due to vectorial addition at the cell surface. 2) Functionally different subpopulations of lymphocytes differ greatly in numbers of exogalactosylatable GlcNAc moieties. 3) Thymocytes contain a much greater proportion of their accessible, terminal GlcNAc residues than do T-lymphocytes or B-lymphocytes from the same animal. 4) Numerous proteins at the cell surfaces of lymphocytes appear to contain one or more O-glycosidically linked GlcNAc monosaccharide moieties, which are readily exogalactosylatable in living cells. However, the bulk of the galactosyltransferable GlcNAc residues are cryptic or localized internally.

Evidence from at least eight independent lines of investigation indicate that we are, in fact, looking at vectorial and not metabolic incorporation of galactose by these exogalactosylation procedures. Perhaps the most convincing is that a 1000-fold excess of unlabeled galactose has no effect on exogalactosylation, and that virtually none of the incorporated [14C]galactose is converted to disaccharide. 4) However, if the cells are first permeabilized with detergent, 60% of the β-eliminatable [14C]hexosamine radioactivity is converted to disaccharide (Fig. 10d). As shown in Fig. 11 (miniprint), HPLC analysis confirms the proportions of this material which are converted to the disaccharide, Galα1-3[14C]GlcNAcitol. Direct compositional analysis of the hydrolyzed, doubly labeled material also confirms the identity of the disaccharide. These findings provide direct evidence that many cell-surface and internal proteins of lymphocytes contain one or more O-glycosidically linked GlcNAc monosaccharide residues. Furthermore, the bulk of these galactosyltransferable terminal GlcNAc residues are either cryptic or found on the inside of the cell.

The finding of only 3% of the incorporated galactose in the internal and external galactosylatable GlcNAc residues, and also to directly determine the identity of the exogalactosylated hexosamine, splenic lymphocytes (a mixture of B- and T-lymphocytes) were preincubated with [1H]glucosamine and subsequently exogalactosylated using unlabeled UDP-galactose. In companion experiments, prelabeled cells were permeabilized with detergent (0.25% NP-40) just prior to galactosylation with UDP-[14C]galactose. Fig. 10 shows the results of Bio-Gel P-4 (-400) chromatography of these galactosylated, [1H]glucosamine-labeled proteins which have been subjected to β-elimination in the presence of excess NaBH4. Table I summarizes several such experiments in quantitative terms. Several conclusions are evident from these data. 1) From 5-11% of the total [1H]glucosamine incorporated by splenic lymphocytes during a 12-h labeling is found in material the size of an N-acetyhexosaminol monosaccharide after β-elimination. Compositional analysis, in fact, demonstrates that at least 61% of this material contains N-acetyl[1H] galactosaminol. 2) β-Eliminated disaccharide accounts for only 1-2% of the incorporated [1H]glucosamine radioactivity in cells which have not been galactosylated. 3) In intact lymphocytes which have been exogalactosylated, only a small percentage (8%) of the β-eliminatable [1H]hexosamine radioactivity is converted to disaccharide. 4) However, if the cells are first permeabilized with detergent, 60% of the β-eliminatable [1H]hexosamine is converted to disaccharide by galactosylation (Fig. 10d). As shown in Fig. 11 (miniprint), HPLC analysis confirms the proportions of this material which are converted to the disaccharide, Galα1-3[14C]GlcNAcitol. Direct compositional analysis of the hydrolyzed, doubly labeled material also confirms the identity of the disaccharide. These findings provide direct evidence that many cell-surface and internal proteins of lymphocytes contain one or more O-glycosidically linked GlcNAc monosaccharide residues. Furthermore, the bulk of these galactosyltransferable terminal GlcNAc residues are either cryptic or found on the inside of the cell.

The finding of only 3% of the incorporated galactose in the
lipid fractions has been observed by others using this approach in other cell types (54). Our extraction experiments suggest that most of the exogalactosylated proteins, which are cell membrane components, are 0-glycosidically linked, has not previously been described.

The quantitative differences in the extent of exogalactosylation observed among the different functional phenotypes of lymphocytes undoubtedly reflects the more general differences that exist in their overall cell-surface saccharide topography, among even morphologically identical, but functionally distinct, cells. Many studies involving lectins (10, 15), galactose oxidase (13, 21), or physical separation techniques (16, 20), have suggested that the cell-surface saccharide topography of lymphocytes correlates with their functional phenotypes. Early studies have suggested that these cell-surface saccharides are important for lymphocyte homing to their correct location in the peripheral lymphoid organs (59, 60). More recent studies have suggested that the complex saccharide structures on lymphoid histocompatibility antigens are involved in their functions at the cellular level (8, 9). Numerous developmental, stage-specific differences in the glycosylation of lymphocyte cell-surface proteins have also been observed (10-21). It is clear that the significance of many of these observed developmental differences must await structural-functional (developmental) analyses at the level of single glycosylation sites on homogeneously purified differentiation antigens (41, 53). In any case, the approach taken in this study does provide a quantitative estimate of the numbers and distribution of terminal GlcNAc residues on the surfaces of lymphocytes of functionally different phenotypes.

We have also attempted to characterize the nature of the oligosaccharide moieties to which the exogenously added galactose is attached. The finding that the bulk of the [3H]galactose was attached O-glycosidically to protein and found on the disaccharide, Galβ1-4GlcNAcitol, after β-elimination was, at first, surprising. The possibility of proteins containing one or more GlcNAc monosaccharides, especially ones which are O-glycosidically linked, has not previously been described. Insensitivity of both the exogalactosylated proteins and their tryptic fragments to digestion with the peptide:N-glycosidase support these β-elimination results. More importantly, they...
also rule out the possibility that the observed galactosylated disaccharide results from alkali-catalyzed cleavage of an unusual glycosidic linkage involving terminal GlcNAc on N-linked oligosaccharides.

Another possibility, namely that the β-elimination conditions might be quantitatively cleaving an internal glycosidic linkage involving an exogalactosylated, nonreducing terminal GlcNAc on a typical O-linked oligosaccharide, seems unlikely based upon kinetic analyses of the β-elimination of galactosylated intact proteins and limit pronase glycopeptides. These kinetic studies indicated that no intermediate oligosaccharides larger than the disaccharide were released, as would be expected in the case of “peeling” (44) or endohydrolytic cleavage of a sugar-sugar glycosidic bond.

We have attempted to eliminate possible sources of artifact in these observations by the use of five independent criteria to establish that the exogalactosylated GlcNAc monosaccharides were originally covalently attached to lymphocyte proteins. The identification of the disaccharide, Galβ1-4GlcNAc, which accounts for greater than 90%, greater than 80%, and at least 49% of the radioactivity from exogalactosylated and β-eliminated B-lymphocytes, T-lymphocytes, and thymocytes, respectively, is not only based upon the chromatographic and compositional criteria, discussed above, but also is consistent with the specificity of the galactosyltransferase employed (25–27, 32). The existence of this apparently unusual monosaccharide structure on many lymphocyte surface proteins and the fact that thymocytes have a much higher proportion of N-linked glycoproteins with terminal, accessible GlcNAc residues may prove to be biologically significant. Even though the bulk of the exogalactosylation is on the O-linked structures, the levels of incorporation are such that it should be possible to examine the N-linked oligosaccharides in more detail, even from B-lymphocytes.

The O-linked GlcNAc monosaccharides may not have been observed in previous metabolic labeling studies because they appear to account for only a small percentage of the incorporated radioactivity and, after β-elimination, they could be easily taken for small amounts of contaminating unincorporated radioactivity or degradation products. If the GlcNAc is attached by the hydroxyl moiety of serine (threonine), and only a fraction of a particular hydroxyamino acid residue in a well studied protein was glucosaminylated, the presence of the GlcNAc could be easily undetected, especially considering the difficulties associated with sequencing at serine or threonine residues. Alternatively, the O-linked GlcNAc residues may not be present on previously well studied glycoproteins.

Earlier studies have also detected single GlcNAc moieties attached to proteins. Hase et al. (61) studied the carbohydrate heterogeneity of Taka-amylase and found a fraction which contained a single GlcNAc as the sugar moiety. These workers concluded that this moiety was the result of endoglycosidase action. Clearly, based upon the peptide:N-glycosidase and β-elimination data, we have eliminated the possibility that the exogalactosylated GlcNAc moieties on lymphocytes arose from the action of either endogenous or exogenous endohexosaminidases acting on N-linked oligosaccharides.

Several very elegant studies have demonstrated the value of highly purified glycosyltransferases in structural-functional problems of receptor function (22) and as probes of saccharide accessibility (56). As vescicular probes of cell-surface saccharide topography, glycosyltransferases have many advantages which make them valuable tools in addition to the conventional approaches using lectin binding. 1) Transferases are extremely specific not only with respect to binding, but also with respect to the reaction they catalyze. 2) Both the donor substrates and the transferase are impermeable to the plasma membrane. 3) Unlike lectins, the glycosyltransferases cause covalent specific modification of the saccharides they interact with, thus allowing radiolabeling, isolation, and characterization of the vectorially labeled glycoconjugates. 4) Covalent modification by exoglycosidase glycosyltransferases allows specific questions of turnover, modification, and saccharide function to be addressed, which would be difficult or impossible by other approaches.

Detailed analyses of oligosaccharide structural microheterogeneity at individual glycosylation sites on important homogeneously purified antigens, which involves the use of metabolic radiolabeling (41, 53), is nicely complemented by similar approaches examining these same glycosylation sites, which have been vectorially radiolabeled at the cell surface. This latter approach has the potential of more accurately detecting subtle topographical alterations or localizations of particular saccharide moieties at the cell surface actually seen by interacting cells, and results in covalent labeling of only those saccharide moieties which are exposed.

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Terminal GlcNAc on Lymphocyte Cell Surfaces

Evolution of Membrane Association of Lymphocyte Enzyme-galactosylated Surface Proteins - To determine whether the labeled, galactosylated proteins were predominately peripheral or integral membrane proteins, a series of extractions were carried out on the labeled lymphocytes. In-glycosylated cells were isolated by centrifugation (as above) and the supernatant was saved. Cell pellets were then extracted at 4°C with 0.01M Tris-HCl, pH 7.5 containing 0.5M NaCl, 5 mM MgCl₂, 0.5 mM CaCl₂, and 7% Apmitin (Sigma). Cells were then washed twice with Ca²⁺/Mg²⁺ free BSS. Cell pellets were then washed in Tris containing Ca²⁺/Mg²⁺ and subsequently re-extracted with 0.01M Tris-HCl, pH 7.5 containing 1% Triton X-100 (Sigma) and 7% Apmitin (Sigma) and 0.5 M NaCl detergent. After washing in the same buffer, the detergent concentration of the extraction buffer was lowered to 0.1% and the extraction repeated. Finally, the cell pellets were solubilized with 0.5M NaCl sample buffer. All fractions were then analyzed for acid insoluble radioactivity and by SDS-PAGE.

Chromatographic Analysis - Fractions from glycosyltransferase labeled cells were precipitated with cold (as above), and digested exhaustively with pre-digested pronase (Calbiochem) as described previously (40). Pronase glycopeptides were analyzed by chromatography on Bio-Rex 70 as described previously (40). Glucosyltransferase was expressed as the total activity of the labeling experiments.

Chromatography of Neuraminidase-Pre-treated Lymphocytes - To determine whether glycosyltransferase INHIBITORS Inhibit the rate at which neuraminidase-treated lymphocytes, and pre-labeled with 16 μg [3H]globoGAL (Amersham). 50 μL of HCl (5 M) was added to cell suspension of 0.5 milli liture in 50 μL of 0.5 M NaCl, 5 mM MgCl₂, 0.5 mM CaCl₂, and 7% Apmitin (Sigma) to the mid point of each experiment. Cells were then extracted with 0.5M Tris-HCl pH 7.5 containing 1% Triton X-100 and 7% Apmitin (Sigma) and 0.5 M NaCl detergent. After washing in the same buffer, the detergent concentration of the extraction buffer was lowered to 0.1% and the extraction repeated. Finally, the cell pellets were solubilized with 0.5M NaCl sample buffer. All fractions were then analyzed for acid insoluble radioactivity and by SDS-PAGE.

Other Analytical Procedures - Sugar composition was determined after acid hydrolysis (45). By HPLC on a reverse-phase column with a water-acetonitrile gradient (45). LPS were extracted by standard Ficol-Hypaque isolation (45). Autoradiography of galactosyltransferase labeled lymphocytes was performed by a previously published procedure (46). Hemolytically active compounds, 0.4% KCl and 0.05% NP-40, were the determined by the Ouchterlony method. Ficol-Hypaque purified, and labeled, 85% galactosylated surface glycoproteins were obtained as described previously (45). All experiments were done independently, as described above.
Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc.

C R Torres and G W Hart

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