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INVESTIGATION OF THE ROLE OF LYMPHOCYTE ECTOGLYCOSYLTRANSFERASES IN BLASTOGENESIS AND CELLULAR INTERACTIONS

by

Robert Otto Endres

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY

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GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Robert Otto Endres

entitled Investigation of the Role of Lymphocyte Ectoglycosyltransferases in Blastogenesis and Cellular Interactions

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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ABSTRACT

Incorporation of carbohydrate from radiolabeled nucleotide sugars into glycoproteins and glycolipids by murine lymphocyte glycosyltransferases was investigated with regard to blastogenesis and whether or not glycosyltransferases occur on the cell surface.

Blastogenesis induced in mouse spleen cells with Concanavalin A (Con A) was characterized by maximal numbers of blast cells and DNA synthesis at 48 hours, and increased incorporation of radiolabeled carbohydrate from UDP-[^{14}C]-galactose, UDP-[^{14}C]-glucose, GDP-[^{14}C]-mannose,and CMP-[^{14}C]-N-acetylneuraminic acid. Utilization of UDP-[^{14}C]-N-acetylglucosamine and UDP-[^{14}C]-N-acetylgalactosamine was not increased.

Paper chromatography of acid soluble material after the one hour assay period revealed that lymphocytes degraded the substrates to the free sugar. Addition of excess unlabeled galactose, mannose, or glucose to assay mixtures with the corresponding radiolabeled nucleotide sugar inhibited uptake of label, indicating that incorporation occurred via the free sugars.

The products of incorporation from UDP-[^{14}C]-galactose, UDP-[^{14}C]-glucose and GDP-[^{14}C]-mannose were acid hydrolyzed and paper chromatography showed that each of the sugars had undergone metabolic conversion. Thin layer chromatography of glycolipids demonstrated that glucosylceramide could be labeled by both UDP-[^{14}C]-galactose and [^{3}H]-galactose. Evidence of sugar interconversion indicated that the free sugar was being transported into the cytoplasm.

The ability of lymphocyte suspensions to degrade nucleotide sugars was found to be very sensitive to inhibition by adenosine-5'-monophosphate (5'-AMP). In the absence of degradation, Con A stimulated lymphocytes could not transport UDP-[^{14}C]-galactose into the acid soluble intracellular pool. The addition of 5'-AMP was then used as an essential criterion for determining cell surface glycosyltransferase activity.

Including 5'-AMP in assays for glycosyltransferase activity in Con A stimulated spleen cells almost eliminated incorporation from UDP-galactose. Galactose could be transferred, however, to an exogenous acceptor. This type of transfer could only be demonstrated with UDP-[^{14}C]-galactose, not [^{3}H]-galactose, suggesting that only extracellular substrate could be utilized. Incorporation from UDP-glucose and CMP-N-acetylneuraminic acid was reduced by 5'-AMP. On the other hand, incorporation from GDP-mannose was unchanged and incorporation from UDP-N-acetylglucosamine was increased. Incorporation from CMP-N-acetyl-neuraminic acid was increased after treatment of the cells with Vibrio cholera neuraminidase.

Lipopolysaccharide (LPS) also induced blastogenesis in spleen cells, but did not increase incorporation from any of the nucleotide sugars tested with 5'-AMP.

In contrast to spleen cells, Con A did increase endogenous galactosyltransferase activity with 5'-AMP in both lymph node and thymus cells. Macrophages exhibited incorporation with 5'-AMP from UDP-galactose, UDP-glucose, UDP-N-acetylglucosamine and GDP-mannose. Sialyltransferase activity on macrophages could only be demonstrated after neuraminidase treatment.

In experiments which utilized Con A, wheat germ agglutinin, Agaricus bisporus lectin and LPS to stimulate spleen and thymus cells, it was found that overall complex carbohydrate synthesis could be stimulated in the absence of blastogenesis, DNA synthesis or agglutination.

Expression of ectomannosyltransferase activity on Con A stimulated spleen cells was found to be higher at the G1/S boundary than during S phase by partial synchronization with hydroxyurea. Mannose was incorporated into two lipid fractions which are similar in their solubility properties to polyisoprenol compounds thought by others to be intermediates in the synthesis of glycoproteins.

The possibility of direct involvement of ectoglycosyltransferases in cellular interactions is discussed with respect to the following: (a) differences of ectoglycosyltransferase expression on various cells, (b) blastogenesis, (c) the cell cycle, and (d) development of the Golgi apparatus.

STATEMENT OF THE PROBLEM

Many different types of cells have been reported to express glycosyltransferase activity on their surfaces. Their role on the cell surface is unknown, but several types of experiments suggest an involvement in contact-mediated cellular interactions. The purpose of this study is to determine if murine lymphocytes express ectoglycosyltransferases and to investigate alterations in this activity during blastogenesis.

INTRODUCTION

Lymphocytes are unique among mammalian cells in that while never an integral part of a solid tissue, they exhibit more different types of functional interaction than any other cell type known. In addition, they are normally quiescent cells which can be stimulated to undergo intense metabolic activity, morphological alteration, and division. These properties make lymphocytes particularly useful for studies of cell interaction mechanisms, induction of cell activation, the regulation of cell growth, and control of gene expression, or differentiation.

Glycosyltransferases are responsible for the synthesis of glycoprotein and glycolipid oligosaccharide structures which are predominantly located on the exterior surface of the plasma membrane. Surface carbohydrates are presently considered to play a dominate role in controlling cell behavior (Crumpton et al., 1975).

Lymphocyte Blastogenesis -- Morphology

Normal unstimulated lymphocytes are 7-8 microns in diameter, with a heterochromatic nucleus and little cytoplasm. The cytoplasm contains predominantly monoribosomes, a few mitochondria, and occasionally some granules. Endoplasmic reticulum is sparse and the Golgi apparatus is barely detectable. Upon stimulation in vitro with mitogenic plant lectins such as phytohemagglutinin (PHA) or concanavalin A (Con A), striking morphological changes occur (Tanaka et al., 1963; Inman and Cooper, 1963; Johnson and Roberts, 1964; Yoffey et al., 1965; Chapman, Elves,

and Gough, 1967; Halpern et al., 1968; Biberfeld, 1971; and Shohat, Janossy, and Dourmashkin, 1973).

These changes can be briefly summarized as follows. Both the cytoplasm and nucleus increase in size with cell diameters reaching 12-18 microns. Some studies describe increased motility, accompanied by cytoplasmic polarization to form a uropod. This lends the lymphocyte its classically referred to "hand-mirror" configuration. The nucleus becomes more euchromatic and complex nucleoli develop. The ribosomal content of the cytoplasm increases, with a greater proportion of polyribosomes seen. Mitochondria become more numerous and some appear swollen. Cytoplasmic granules increase in number and variety of staining characteristics. Pinocytic vesicles appear. Although little or no increase in the amount of endoplasmic reticulum occurs in PHA stimulated cells, the Golgi apparatus becomes highly developed and centrioles form near the Golgi region. Mitotic figures are first seen after one to two days in culture, reaching a maximum number by 48 hours for mouse lymphocytes and 72 hours for human lymphoctyes.

Lymphocyte Blastogenesis -- Metabolism

Blastogenic transformation in lymphocytes is accompanied by biochemical changes in nuclear chromatin-associated proteins (Kleinsmith, Allfrey, and Mirsky, 1966; Pogo, Allfrey, and Mirsky, 1966; and Dobozy, Hunyadi, and Simon, 1970), leading to increased RNA synthesis (Epstein and Stohlman, 1964; Cooper and Rubin, 1965; Rubin and Cooper, 1965; and Cooper, 1970) and protein synthesis (Asofsky and Oppenheim, 1966; Forbes and Henderson, 1966; Parenti et al., 1966; and Ling, 1968b), followed at

24-72 hours by DNA synthesis (Bender and Prescott, 1962; McIntyre and Ebaugh, 1962; Cooper, Barkhan, and Hale, 1963; and Ling, 1968a). Glycogen and lipid stores appear early, but are consumed during the course of transformation (Quaglino, Hayhoe, and Flemans, 1962, Tanaka et al., 1963; and Biberfeld, 1971). The hydrolytic enzyme content of lysosomes increases (Hirschhorn et al., 1965; Gough and Elves, 1967; and Hirschhorn, Hirschhorn, and Weissmann, 1967).

The energy needed for blastogenesis is predominantly supplied by increased glycolysis (Cooper, Barkhan, and Hale, 1963; Hedeskov, 1968; and Rabinowitz, Schimo, and Wilhite, 1968), although oxidative phosphorylation is also necessary (Hedeskov, 1968; Roos and Loos, 1970; and Roos and Loos, 1973). Hexose monophosphate shunt activity increases to provide reducing equivalents and biosynthesic intermediates (MacHaffie and Wang, 1967; Hedeskov, 1968; and Sagone, LoBuglio and Balcerzak, 1974).

Lymphocyte Blastogenesis -- Membrane Alterations

Of primary interest to this study are the plasma membrane changes which occur, with their implications for cell functioning and interaction. The earliest changes observed are (a) an increased transport of potassium ions, correlating with increased Na^+-K^+ dependent adenosine triphosphatase activity (Quastel and Kaplan, 1970; Averdunk, 1972; Novogrodsky, 1972; and Averdunk and Lauf, 1975), (b) transiently increased adenylcyclase (Smith et al., 1971; and Krishnaraj and Talwar, 1973), and possibly guanylcyclase (Hadden et al., 1972; and Wedner, Dankner, and Parker, 1975) activities, (c) increased transport of the glucose analogues --

3-O-methylglucose (Peters and Hausen, 1971) and α-methylglucoside (Averdunk, 1972), (d) increased incorporation of $^{32}PO_4$ into phosphatidylinositol (Fisher and Mueller, 1968; Fisher and Mueller, 1971; Lucas, Shohet, and Merler, 1971; and Betel, Martijnse and van den Berg, 1974), and (e) redistribution of surface receptors into patches, then "caps," followed by internalization (Smith and Hollers, 1970; and Loor, 1973).

Changes in these parameters, compared to cells incubated without mitogen treatment, are detectable before 5 or 10 minutes after addition of the mitogen. Other changes which are detected soon thereafter are (a) increased transport of calcium ions (Allwood et al., 1971; and Whitney and Sutherland, 1972), uridine (Peters and Hausen, 1971), and aminoisobutyric acid, an amino acid analogue (Averdunk, 1972; and van den Berg and Betel, 1971), (b) increased synthesis of phospholipids (Kay, 1968; Fisher and Mueller, 1968; Fisher and Mueller, 1971; Resch et al., 1971; Resch et al., 1972; Resch and Ferber, 1972), glycolipids (Inouye, Handa, and Osawa, 1974), and neutral lipids (Kay, 1968; Resch and Ferber, 1972; and Inouye, Handa, and Osawa, 1974), (c) enhanced incorporation of glucosamine into surface glycoproteins (Hayden, Crowley, and Jamieson, 1970), and (d) enhanced turnover of at least one membrane protein (Schmidt-Ullrich, Wallach, and Ferber, 1974).

Cellular Interactions of Lymphocytes

Circulating lymphocytes seem to have a strong attraction for the unique endothelial cells of postcapillary venules in lymphatic tissue (Marchesi and Gowans, 1964; Claesson, Jorgensen, and Ropke, 1971; Messier and Sainte-Marie, 1972; and Schoefl, 1972). The localized transmigration

through these vessel walls seems to be related to control of lymphocyte distribution between tissues and blood. Carbohydrates of the lymphocyte surface are thought to be involved in this control (Gesner and Ginsburg, 1964). Lymphocytes are also capable of mass transmigration of vessel walls in other tissues during inflammation (Astrom, Webster, and Arnason, 1968).

In tissue explants, motile lymphocytes have been observed to have a particular affinity for adhering to megakaryocytes and crawling over macrophages and mitotic cells in telophase (Humble, Hayne, and Pulvertaft, 1956).

Infiltration of malignant tumors and allografts of skin has been demonstrated to occur in vivo (Murphy, 1926; Berg, 1959; and Rothwell and Papdimitriou, 1972). Lymphocytes show several distinct types of physical interaction with tumor cells in tissue explants (Humble et al., 1956; and Sherwin and Richters, 1972). Sherwin and Richters classified these interactions into eight categories: clumping, clustering, congregation, polesis, peripolesis, pedopolesis, emperipolesis and mechanoclasmatosis (destruction of the tumor cell). When lymphocytes are sensitized in vivo and exposed to tumor cells or allogeneic cells in vitro, they are capable of antigenically specific adherence (Brondz, 1968; Brondz and Goldberg, 1970; and Golstein, Svedmyr and Wigzell, 1971) resulting in the generation of cytotoxic lymphoblasts which can kill a monolayer of target cells (Govaerts, 1960; Alexander, Connell, and Mikulska, 1966; and Rosenau and Morton, 1966).

Clustering of lymphocytes around macrophages is seen in vivo in lymph nodes responding to immunization (Andre-Schwartz, 1964; Schoenberg

et al., 1964; and Miller and Avrameas, 1971). In vitro, lymphocyte-macrophage interaction is important in the induction of antibody synthesis (Fishman and Adler, 1963; and Mosier, 1969), in the blastogenic response to mitogens or antigens (Hersh and Harris, 1968), and in the mixed leukocyte reaction (McFarland and Heilman, 1965; McFarland, Heilman, and Moorhead, 1966; and Gordon, 1968). Lymphocytes have been observed in prolonged migration over the macrophage surface (Sharp and Burwell, 1960), or attached for extended periods in clusters on a central macrophage (McFarland et al., 1966; Lamvik, 1969; and Mosier, 1969).

Physical interactions between lymphocytes and macrophages also take place in the absence of specific antigen, and may be related to maintenance of lymphocyte viability (Chen and Hirsch, 1972; and Pierce et al., 1974). Admixture of thymocytes with macrophages or thymus epithelial cells in vitro has been shown to induce functional and antigenic maturation of the thymocytes (Mosier and Pierce, 1972).

In addition to this array of interactions between lymphocytes and other cells, subpopulations of lymphocytes are also known to interact. This was first discovered when Claman, Chaperon, and Triplett (1966a and 1966b) found that neither thymus nor bone marrow cells alone could reconstitute an X-irradiated mouse for antibody production, but that both combined could. Since this finding, many types of lymphocyte-lymphocyte interaction have been found. One type has negative effects on immune responses and is called suppression. Although at present suppression is only an operational term describing a phenomenon, it appears that a subpopulation of thymus derived cells can specifically inhibit the response

to a given antigen (Gershon and Kondo, 1970; 1971; for review, see Dutton, 1975).

In allogeneic responses against tissues or cells, subpopulations of thymus derived lymphocytes have been found which can synergize to enhance the response. Synergy seems to be important for both the proliferative and cytotoxic phases of the in vitro mixed lymphocyte reaction (Cohen and Howe, 1973; Tigelaar and Feldmann, 1973; and Wagner, 1973), as well as the in vivo graft versus host reaction (Cantor, Asofsky, and Talal, 1970; Cantor and Asofsky, 1970; 1972).

Fundamentals of Glycosyltransferases

Glycosyltransferases catalyze the general reaction:

$$\text{Nucleotide-monosaccharide} + \text{Acceptor} \xrightarrow{Mn^{+2} \text{ or } Mg^{+2}} \text{Acceptor-monosaccharide} + \text{Nucleotide}$$

where the acceptor is usually a glycoprotein or glycolipid with an incomplete oligosaccharide moiety. For each substrate, a given sugar must be activated by a specific nucleotide, e.g., uridine diphosphate for galactose. It is thought that some glycosyltransferases are not only specific for the nucleotide sugar, but also for the type of linkage and position in the oligosaccharide chain.

Glycosyltransferases are predominantly located in the Golgi apparatus (Schachter et al., 1970; Dauwalder, Whaley and Kephart, 1972; and Northcote, 1972), where they help synthesize glycoproteins and glycolipids for export (Neutra and LeBlond, 1966a; 1966b; Horwitz and Dorfman, 1968; and Whur, Herscovics, and LeBlond, 1969) or for insertion into the plasma membrane (Bennett, LeBlond and Haddad, 1974; and Atkinson, 1975). The

structures constructed by glycosyltransferases are capable of conferring powerful antigenic specificities on the molecule and cell they are attached to. The classic example is the AB pair of human blood group antigens, which are oligosaccharides differing only in their terminal sugar (Watkins, 1966).

Cell-associated complex carbohydrates are preferentially located on the hydrophylic exterior of the plasma membrane (Hirano et al., 1972; Parsons and Subjeck, 1972; and Steck and Dawson, 1974). Here they play a major role in determining cellular behavior. Cell surface oligosaccharides seem to be involved in intercellular adhesion and control of adhesive specificity (Oppenheimer et al., 1969; Roth, McGuire, and Roseman, 1971a; Chipowski, Lee, and Roseman, 1973; and Balsamo and Lilien, 1975). They can serve as cell surface antigens (Watkins, 1966; Makita, Susuki, and Yosizawa, 1966; Bona et al., 1972; Vitetta, Boyse, and Uhr, 1973; and Trowbridge and Hyman, 1975) and mitogenic lectin receptors (Sharon and Lis, 1972). Surface complex carbohydrate alterations are known to occur in neoplastic transformation and have been correlated with the loss of growth control (Pollack and Burger, 1969; Brady and Fishman, 1974; Gahmberg, Kiehn, and Hakomori, 1974; Hakomori et al., 1974; Steiner et al., 1974; Warren et al., 1974; and Hakomori, 1975).

Ectoglycosyltransferases

Although they are mainly located in the Golgi apparatus, glycosyltransferases are now thought to also be present on cell surfaces as ectoenzymes (Barber and Jamieson, 1971; Bosmann, 1971; Roth, McGuire, and Roseman, 1971b; Roth and White, 1972; Arnold, Hommel and Risse, 1973;

and Yogeeswaran, Laine, and Hakomori, 1974). Ectoenzymes are defined as plasma membrane enzymes whose active sites face the exterior (DePierre and Karnovsky, 1973). It has been postulated that ectoglycosyltransferases play a direct role in cell adhesion (Roseman, 1970; Roth et al., 1971a; and Roseman, 1974) and interaction (Roth et al., 1971b; and Roth, 1973) by binding acceptor molecules on adjacent cells.

Evidence that they may be involved in cellular interactions comes from several areas of investigation (reviewed by Shur and Roth, 1975). Weiser (1973) studied a graded distribution of rat intestinal epithelial cells, ranging from undifferentiated crypt cells to mature villus cells. He found up to tenfold higher endogenous glycosyltransferase activity (presumed to be on the cell surface) with GDP-mannose, GDP-fucose, UDP-N-acetylglucosamine, UDP-galactose and UDP-glucose on crypt cells as opposed to villus cells. Sialyl transferase activity was preferentially expressed on the villus cells.

Two reports have shown surface activity to vary with respect to the mitotic cycle. Webb and Roth (1974) found that 3T3 cells in mitosis did not require contact for demonstration of maximum galactosyltransferase activity, whereas interphase cells did. Bosmann (1974) measured incorporation from CMP-N-acetylneuraminic acid, UDP-galactose and UDP-N-acetylglucosamine onto exogenous acceptors in synchronized L5178Y cells. Incorporation from all three was highest in the S phase, and lowest during mitosis.

Several investigators have found altered surface glycosyltransferase activity in transformed cells as compared to their normal counterparts (Bosmann, 1972; LaMont, Weiser, and Isselbacher, 1974; Patt and

Grimes, 1974; Yogeeswaran et al., 1974; and Patt, Van Nest, and Grimes, 1975). The alterations are inconsistent, though, in that the activities may be higher or lower on transformed cells. In addition, normal cells in culture exhibit decreased ectoglycosyltransferase activity in confluent as compared to sparse conditions, while transformed cells exhibit the same activity at all cell densities (Bosmann, 1972; and Roth and White, 1972).

Glycosyltransferase assays are influenced by the amount of active enzyme present, the availability of incomplete acceptors, and the availability of intact nucleotide sugar substrate. Evans (1974) demonstrated a nucleotide pyrophosphatase ectoenzyme on rat liver cell plasma membranes. It was suggested that this enzyme would rapidly degrade the nucleotide sugar substrates used to measure ectoglycosyltransferase activity, allowing transport of the free sugar and incorporation via the Golgi enzymes. Deppert, Werchau, and Walter (1974) found this to be true for BHK cells, although Patt and coworkers could find no evidence of significant substrate degradation to the free sugar using 3T3 fibroblasts (Patt and Grimes, 1974; Patt et al., 1976).

Although the functions of cell surface carbohydrates and ectoglycosyltransferase are unknown, the evidence is strong that they are involved in many aspects of cell interactions.

MATERIALS AND METHODS

Reagents and Isotopes

Plant lectins used in this study were concanavalin A (Calbiochem, La Jolla, Ca.), phytohemagglutinin (Difco, Detroit, Mich.), wheat germ agglutinin (Sigma Chemical Co., St. Louis, Mo.), and a lectin from the mushroom Agaricus bisporus which was kindly donated by Dr. Harvey J. Sage from the Department of Biochemistry, Duke University Medical Center, Durham, N. C.

Lipopolysaccharide (from Escherichia coli, serotype 0127:B8), adenosine-5'-monophosphoric acid, and hydroxyurea were obtained from Sigma Chemical Co., St. Louis, Mo.

Isotopes used were thymidine [methyl-^{3}H], 20 Ci/mmole; uridine [5-^{3}H], 26 Ci/mmole; [^{3}H]-galactose, 250 mCi/mmole; [^{3}H]-l-mannose, 326 mCi/mmole; uridine diphosphate-[^{14}C]-galactose, 257-281 mCi/mmole; uridine diphosphate-[^{14}C]-glucose, 224-227 mCi/mmole; uridine diphosphate-[^{14}C]-N-acetylgalactosamine, 51.5 mCi/mmole; uridine diphosphate-[^{14}C]-N-acetylglucosamine, 56.5 mCi/mmole; guanidine diphosphate-[^{14}C]-mannose, 221-276 mCi/mmole; and cytidine monophosphate-[^{14}C]-N-acetyl-neuraminic acid, 217 mCi/mmole. All isotopes were purchased from New England Nuclear, Boston, Mass.

Mice

Mouse strains used in this study were inbred C3H, BALB/C and C57B1/6. They were housed and bred in the Division of Animal Resources,

Arizona Medical Center, Tucson, Arizona. All mice used for obtaining thymus cells were 3-5 weeks of age, and all mice used for obtaining spleen, lymph node, or peritoneal exudate cells were 1-5 months of age. Mice of only one sex were used for each experiment.

Cell Cultures

Depending on the experiment, spleens, thymuses, or lymph nodes were excised under sterile conditions and placed in Hank's balanced salts solution supplemented with 0.6% dextran (HBSS + dextran). When lymph nodes were used, the particular nodes chosen were usually the mesenteric, inguinal, lumbar, axillary, and superficial cervical nodes. Organs were teased with scalpels, the connective tissue was removed and the leukocytes were dispersed by expressing through a 20 gauge needle with mild force.

After washing twice with HBSS + dextran, cells were suspended in RPMI medium supplemented with 5% fresh human serum, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. Cultures were set up at 5 x 10^6 mononuclear cells per ml in a volume of 2 ml in Falcon 17x100 mm tissue culture tubes. In cultures stimulated with concanavalin A, the lectin was added to a final concentration of 10 µg/ml. Cultures were incubated at 37°C in a humidified atmosphere flushed with CO_2 at 0.2 liters/min.

Viability was determined by trypan blue dye exclusion. Percent blast cells was determined by counting in 3% acetic acid + gentian violet, which selectively lysed erythrocytes. Cells counted as blasts had enlarged nuclei and an increased cytoplasm/nucleus ratio. Macrophages

could be distinguished from blast cells by the presence of numerous cytoplasmic vacuoles and by the lack of an easily discernable nucleus, as viewed in this diluting fluid.

For tests on macrophages, peritoneal exudate cells were harvested with HBSS + dextran from mice injected intraperitoneally with 2 ml sterile thioglycollate broth three days previously. The cells were washed twice, and dispensed onto collagen gel layers (described in the next section) in 150x15 mm Falcon tissue culture dishes, using RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin, and streptomycin. After three to six hours, each dish was gently rocked to remove nonadherent cells. The medium and nonadherent cells were then discarded, and replaced with fresh medium. These cultures were then incubated at 37°C in a CO_2 atmosphere for 2-5 days to allow for the death of adherent polymorphonuclear cells. At the end of that time, monolayers were washed twice with Tris buffered saline (TBS) at pH 7.4, and incubated with 4 ml collagenase solution (50 U/ml, Worthington Biochemical Corp., Freehold, N. J.) for 20 min at 37°C. This dissolved the collagen gel layer and released all adherent cells with better than 99% viability. These cells are referred to as purified macrophages in the text. Purified macrophages were washed twice to remove the collagenase before testing.

Preparation of the Collagen Gel Layers

Collagen was extracted from mouse tail tendons by the method of Ehrmam and Gey (1956). Eight to twelve tails were skinned and soaked in 95% ethanol for 1-4 hr, then washed with sterile water. Tendons were then separated, chopped into small pieces with scissors and dropped into cold

0.1% acetic acid (100-120 ml). This was stored at 4°C for 24 hr, spun at 1500 g for 2 hr, and the clear, viscous supernatant solution was collected. Then fresh acid was added (about 2/3 the amount removed) and the process was repeated twice more. The third storage period was 48 hr rather than 24 hr.

The resulting 100-150 ml of collagen solution was then dialyzed against 4 liters of deionized water for six hr. In this state, the solution could be stored for months at 4°C or frozen at -20°C.

Collagen gel layers were formed when a thin layer of solution in tissue culture dishes was exposed to ammonia vapor. To make the clear, firm gel suitable for supporting viable cells, it was soaked with two changes of sterile water containing antibiotics over a 24 hr period. This was then replaced with an amount of 2X HBSS equal to the volume of the collagen, plus a surplus of 1X HBSS for 24 hr. This served to bring the gel fluid phase to isotonicity. Gels were incubated a further 24 hr with the complete medium to be used for culturing cells. Fresh medium was added again with the cells.

Assay for Incorporation of Carbohydrate into Glycoproteins and Glycolipids

This procedure is a modification of that described by Patt and Grimes (1974). At the end of the culture period, cells were pooled, washed twice in TBS (pH 7.4) and resuspended. 100 µl aliquots containing approximately 2×10^6 cells (usually 0.1 mg protein) were dispensed to test tubes containing 10 µl of the appropriate nucleotide sugar substrate (100 pmole) and 10 µl of either 0.1 M $MnCl_2$ or 0.3 M $MgCl_2$. Mn^{+2} ion was used with UDP-[^{14}C]-galactose, UDP-[^{14}C]-N-acetylgalactosamine, and

CMP-[^{14}C]-N-acetylneuraminic acid. Mg^{+2} ion was used with UDP-[^{14}C]-glucose, UDP-[^{14}C]-N-acetylglucosamine, and GDP-[^{14}C]-mannose. For assay of cell surface activity, 10 µl of 65 mM adenosine-5'-monophosphate was added (final concentration 5 mM) to inhibit pyrophosphatase degradation of substrates (see Results). Incubations with boiled cells were always included for subtraction of background radioactivity.

After a one hr incubation at 37°C, reactions were terminated with cold 10% trichloroacetic acid (TCA) and the precipitates collected on 2.4 cm Whatman glass fiber filter pads. These were washed twice with 5% TCA, dried, and placed in scintillation vials. Glycolipids were extracted by two washes with 2 ml chloroform : methanol (2:1), leaving glycoproteins on the pads. Both washes were pooled in a second vial and evaporated to dryness. Radioactivity was measured in a scintillation spectrometer using a cocktail consisting of toluene and Omnifluor (New England Nuclear, Boston, Mass.).

Assay for DNA Synthesis

Cultures to be assayed for DNA synthesis were set up in one ml volumes in Falcon 12x75 mm culture tubes. 1.0 µCi of thymidine [methyl-^{3}H] was added 4-6 hr before the end of the culture period. Using a modification of the procedure described by Mans and Novelli (1961), 200 µl samples were then applied to 2.3 cm Whatman paper filter pads and allowed to dry. Pads were soaked for 1-4 hr in cold 10% TCA, 15 min in 5% TCA at room temperature, and washed once with 95% ethanol, once with acetone. When dry, radioactivity was measured by liquid scintillation spectrometry as described above.

Determination of Substrate Degradation

Cell aliquots were incubated with radioactive nucleotide sugar substrates as described for the carbohydrate incorporation assay procedure. At the end of the incubation, reactions were stopped with cold 10% TCA and pelleted. The supernates were neutralized with 2.5 N NaOH, and made 20-25 mM in ethylene diamine tetraacetate to remove the Mn^{+2} or Mg^{+2} ions. These were subjected to descending paper chromatography on 1x20 inch Whatman No. 1 paper strips in a solvent of ammonium acetate (1.0 M, pH 3.6) : 95% ethanol (30:75). The strips were allowed to develop 17-20 hr, dried, cut into one inch sections and radioactivity was measured by liquid scintillation. Unlabeled standards used were the nucleotide sugar, sugar phosphate, and free sugar corresponding to the given radioactive substrate. These were detected by a periodate-permanganate spray (Limieux and Bauer, 1954).

Neutral Sugar Analysis

After cell suspensions were incubated with the radioactive nucleotide sugar, the labeled products were precipitated and washed with cold 10% TCA. Acid hydrolysis was carried out in sealed tubes with one ml of 2N triflouroacetic acid at 120°C for 90 min. Samples were then dried at 40°C with filtered air, and reconstituted with distilled water. Neutral sugars were separated by chromatography on Whatman No. 1 paper strips using a solvent system of isobutyric acid : ammonia : water (54:4:49).

Preparation of Exogenous Acceptors

Asialo-fetuin was prepared by incubating 500 mg fetuin (Type II, Sigma Chemical Co., St. Louis, Mo.) in 40 ml of 0.05 N H_2SO_4 at 80°C for one hr. The mixture was then cooled and neutralized with 1.0 N NaOH. Finally, the mixture was dialyzed against several changes of distilled water and lyophylized. The asialo-fetuin was stored either as the powder at 4°C or dissolved in phosphate buffered saline (pH 7.4) at 10 mg/ml and frozen at -20°C.

Asialo-agalacto-fetuin was then prepared by dissolving 200 mg asialo-fetuin and 40 mg almond emulsin (β-glucosidase, Sigma Chemical Co., St. Louis, Mo.) in 80 ml of 0.1 M sodium acetate buffer, pH 5.0. The mixture was then incubated at 37°C for 48 hr. After digestion, the mixture was washed through a 2.5x25 cm column of DE-52 anion exchanger (W. & R. Balston, Ltd., Maidstone, Kent.). The column was preequilibrated with 0.02 M H_2KPO_4 adjusted with NaOH to pH 7.0. After the digest mixture had been applied to the column, and washed with excess buffer, the column was eluted with a linear gradient of 0-1 M KCl in buffer. The enzyme and free galactose eluted in the excluded volume, and the asialo-agalacto-fetuin eluted at a salt concentration of 0.3 M. Fractions containing the acceptor were then dialyzed against distilled water and lyophylized. The powder was either stored as such at 4°C or dissolved in phosphate buffered saline (pH 7.4) at 10 mg/ml and frozen at -20°C.

This method of preparation was previously described by Grimes (1973).

RESULTS

Characterization of Mouse Spleen Cell Response to Concanavalin A

When mouse spleen cells were incubated with 10 μg/ml Concanavalin A (Con A), the number of blast cells present rose to a maximum of about 60% at two and three days (Fig. 1, left). Control cultures contained 3-4% blast cells and that number did not increase. The rate of DNA synthesis, as measured by incorporation of thymidine [methyl-^{3}H] was maximal at two days (Fig. 1, right). The low level of incorporation seen in control cultures remained low. Viability of both Con A and control cultures dropped from an initial value of 85-95% to 50-60% by day four.

Glycosyltransferase activity associated with cell suspensions was measured by their ability to incorporate radioactive carbohydrate from labeled nucleotide sugars into both glycoproteins and glycolipids. To determine changes in incorporation as a function of time after addition of Con A, cells were assayed every 24 hr with UDP-[^{14}C]-galactose (Fig. 2). Incorporation was found to be stimulated by Con A. The greatest activity was seen at 24 and 48 hr, rising somewhat earlier than DNA synthesis. Glycoproteins and glycolipids were labeled in roughly equivalent proportions. In the same experiment, incorporation of [^{3}H]-galactose, which must be metabolized intracellularly for incorporation, followed an identical pattern.

Fig. 1. Time course of Con A stimulated blastogenesis.

Solid lines, mouse spleen cells incubated for 48 hr with Con A; dashed lines, control cells. Shown on the left, the percent blast cells were counted by phase contrast microscopy on cell aliquots identical to those assayed for incorporation every 24 hr. Each value is the mean of three separate experiments $\pm$ the standard deviation. Shown on the right, DNA synthesis was assayed every 24 hr by adding 1.0 µCi of thymidine [methyl-^{3}H] to one ml cultures containing 5 x 10^6 cells originally. After 4 hr incubation, 200 µl aliquots were applied to 2.3 cm Whatman filter pads, dried, soaked in cold 10% TCA for 4 hr, then 5% TCA for 15 min, and finally washed with 95% ethanol and acetone. Pads were counted by liquid scintillation spectrometry. Each value is the mean $\pm$ standard deviation of duplicate samples from duplicate tubes.

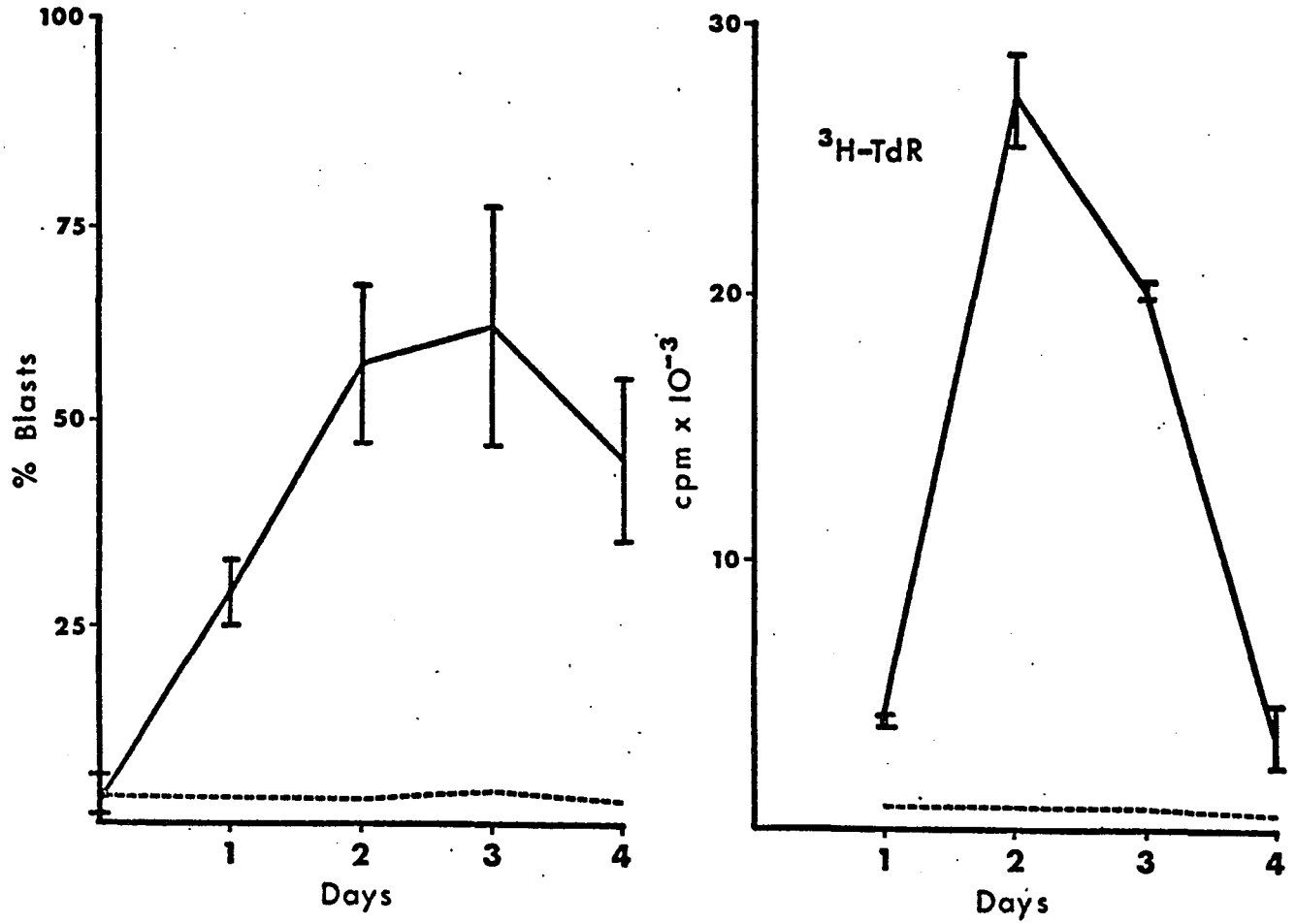


Fig. 1. Time course of Con A stimulated blastogenesis.

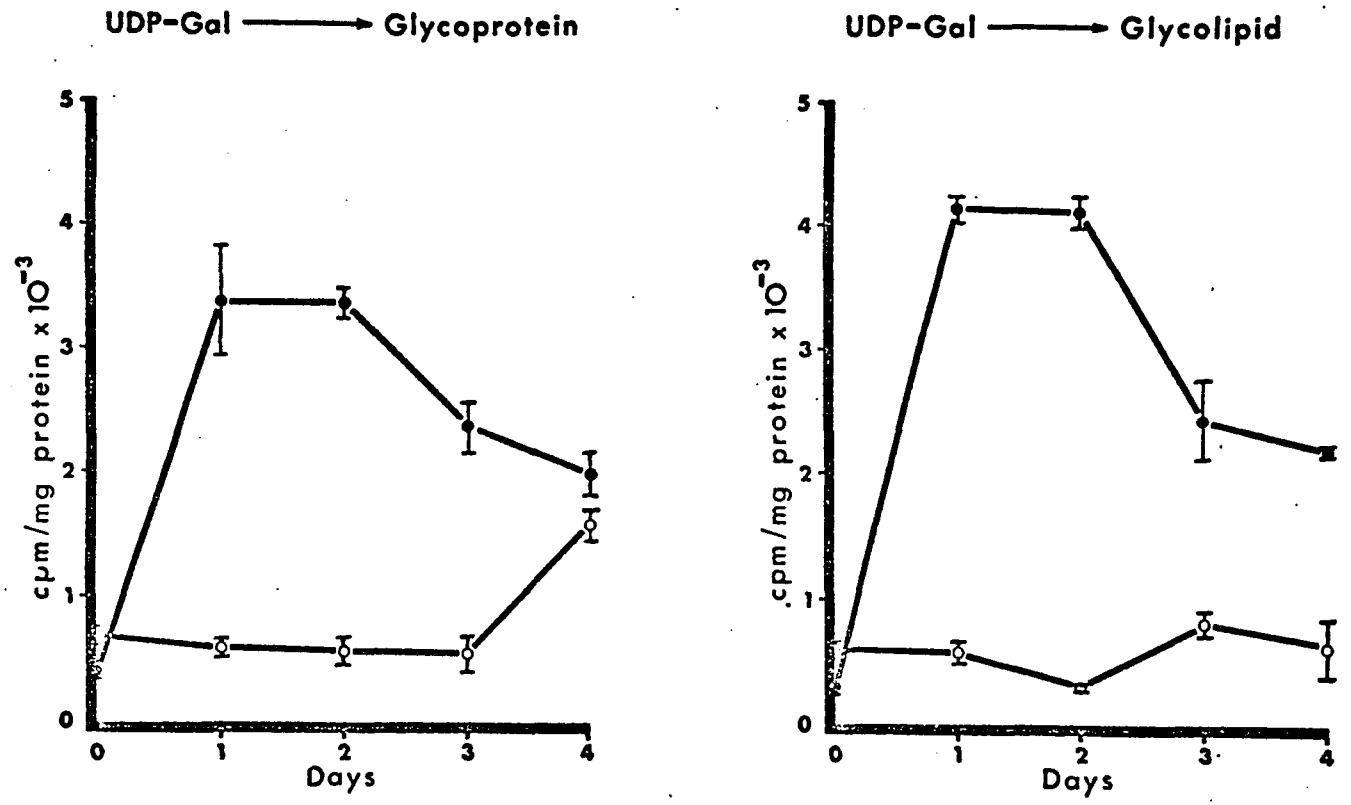


Fig. 2. Time course of incorporation of label from UDP-[^{14}C]-galactose by Con A stimulated mouse spleen cells.

Aliquots were assayed every 24 hr for incorporation into both glycoproteins (left) and glycolipids (right). Closed circles, Con A; open circles, control. Each value is the mean of duplicate samples ± the range.

Fig. 3 shows the results obtained when mouse spleen cells, incubated for 48 hr with Con A, were assayed for the ability to incorporate carbohydrate from other nucleotide sugars. Incorporation of label from UDP-[^{14}C]-galactose, UDP-[^{14}C]-glucose, and GDP-[^{14}C]-mannose into both glycoproteins and glycolipids was increased by Con A stimulation. Incorporation from CMP-[^{14}C]-N-acetylneuraminic acid and GDP-[^{14}C]-fucose (not shown) was observed only in glycoproteins, and was also increased. Incorporation of label from UDP-[^{14}C]-N-acetylgalactosamine and UDP-[^{14}C]-N-acetylglucosamine was low and did not increase after incubation with Con A.

Substrate Degradation

To investigate breakdown of nucleotide sugars in Con A stimulated lymphocytes, several types of experiments were carried out. The most direct evidence that intact lymphocytes degrade nucleotide sugars is seen in Fig. 4. Paper chromatography was used to separate TCA soluble isotope remaining after various fractions of the one hour assay period. It can be seen that UDP-galactose was rapidly hydrolyzed to galactose-1-phosphate and free galactose. Incorporation into TCA insoluble material increased in a linear fashion, but only after a five minute lag period, coinciding with the increase in free galactose. The appearance of free galactose suggests the action of a hexose monophosphatase as well as the nucleotide pyrophosphatase described by Evans (1974).

All nucleotide sugar substrates tested were found to be degraded by both Con A stimulated spleen cells and control cells (see Table 1). Stimulation by Con A enhanced the extent of degradation compared to

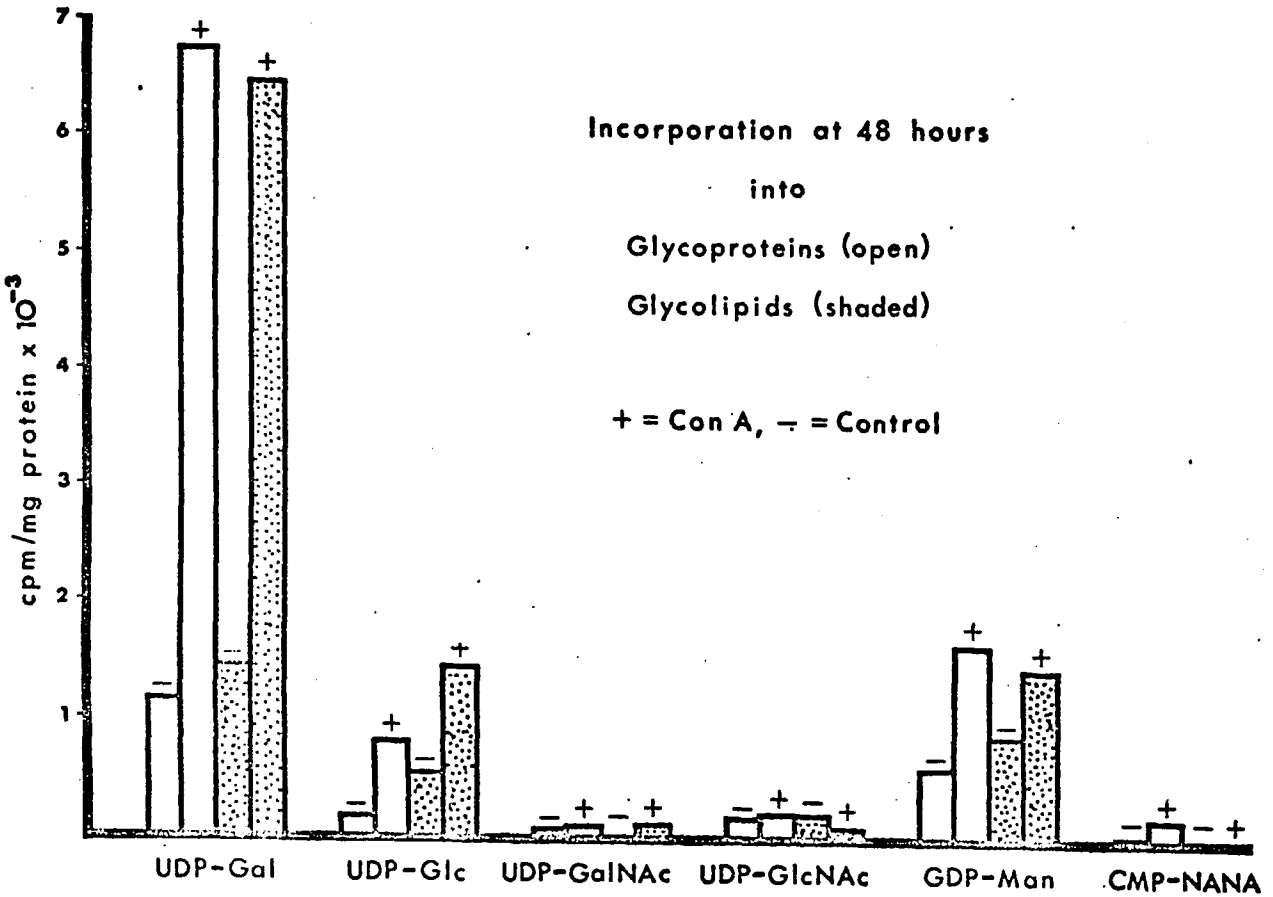


Fig. 3. Incorporation of labeled sugars from nucleotide sugar substrates into mouse spleen cells incubated for 48 hr with Con A.

Incorporation into glycoproteins and glycolipids was assayed in each sample. Each value is the mean of duplicate samples and the ranges were 5-10% of the means.

Fig. 4. Time course of hydrolysis of UDP-[^{14}C]-galactose by Con A stimulated lymphocytes.

At the times indicated, duplicate tubes were terminated with the addition of cold 10% TCA. TCA precipitable material was pelleted and aliquots of the supernates were chromatographed on Whatman No. 1 paper in a solvent of ammonium acetate (1.0 M, pH 3.6) : 95% ethanol (30:75) for 17-20 hr as described in Materials and Methods. Unlabeled UDP-galactose, galactose-1-phosphate and galactose were used for identification of radioactive peaks. The upper figure indicates the relative amount of label in each sugar derivative at each time tested. (?) indicates label in an unidentified peak from the chromatography. The lower figure shows total incorporation into the TCA pellets from the same experiment. Glycoproteins and glycolipids were not separated.

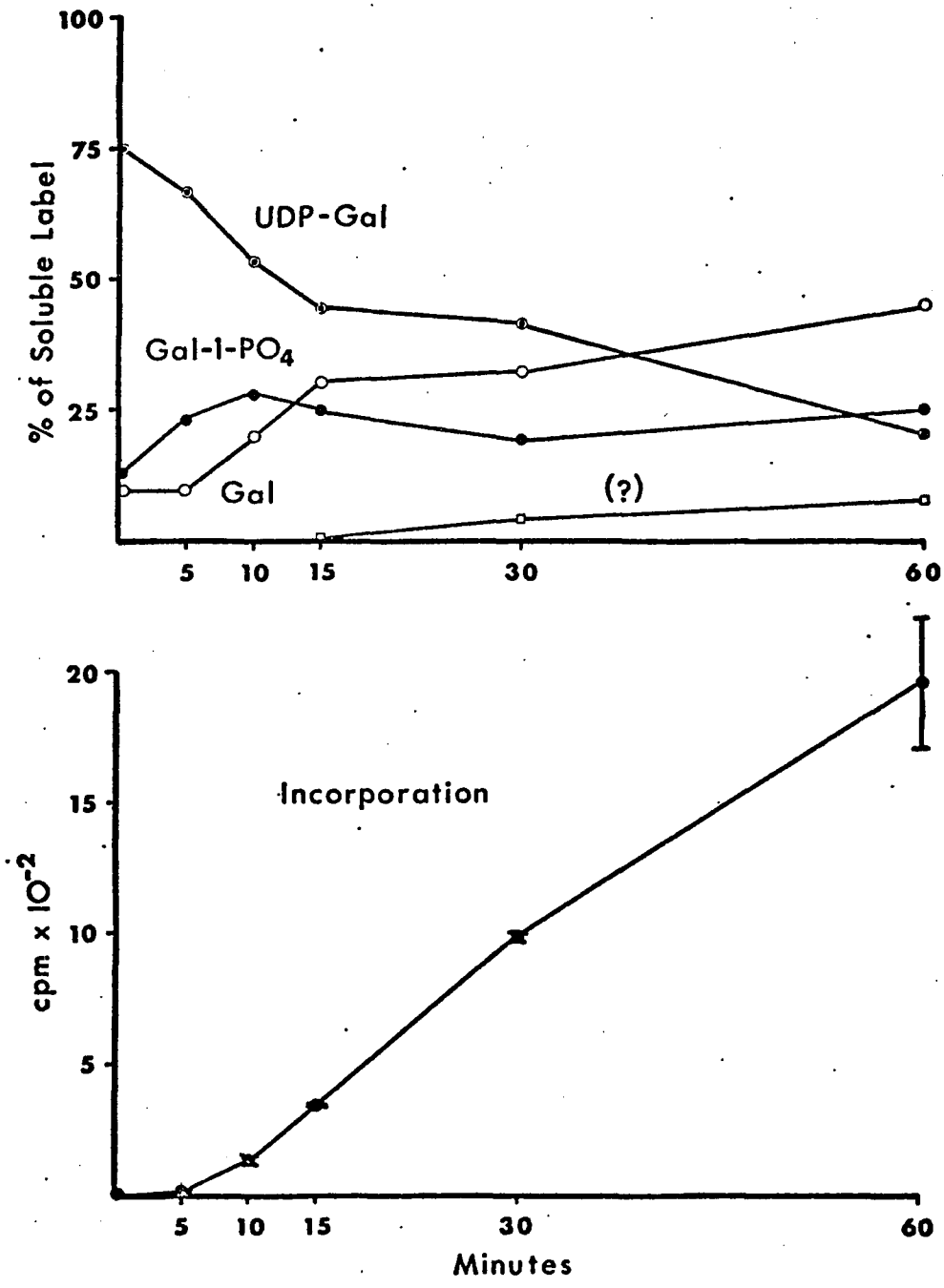


Fig. 4. Time course of hydrolysis of UDP-[^{14}C]-galactose by Con A stimulated lymphocytes.

Table 1. Percent of label left in supernate after one hour.[a]

Substrate	Con A	Nucleotide Sugar	Sugar-PO_4	Free Sugar	Unidentified Products
UDP-Gal	+	10	38	49	3
	-	9	51	39	1
UDP-Glc	+	23	51	22	3
	-	28	50	20	2
UDP-GalNAc	+	6	9	86	0
	-	36	38	26	0
UDP-GlcNAc	+	18	21	61	0
	-	59	27	15	0
GDP-Man	+	22	11	40	28
	-	52	30	18	0
CMP-NANA	+	43	0	57	0
	-	57	0	43	0

a. Substrate Degradation -- After 48 hours of stimulation with Con A, aliquots of stimulated (+) and control (-) cells were incubated with the labeled substrates shown in the left column. (The ^{14}C was located in the sugar moiety of all substrates.) After 60 minutes, the reactions were stopped with cold 10% TCA and pelleted. The supernates were subjected to paper chromatography for 17-20 hours on Whatman No. 1 paper in a solvent of ammonium acetate (1.0M, pH 3.6) : 95% ethanol (30:75). Unlabeled standards used were the nucleotide-sugar, sugar-phosphate and free sugar corresponding to the given substrate.

control, which could be a possible factor in the increased incorporation of label from some of the substrates.

Additional evidence for substrate degradation and uptake of the free sugar was found in analysis of the labeled carbohydrate incorporated into TCA insoluble products. If the free sugar were actually used intracellularly, it would be expected to undergo epimerization to other sugars which could also appear in acid insoluble products.

In studies to determine substrate stability, mouse spleen cells were stimulated for 48 hr with Con A, and incubated with UDP-[^{14}C]-galactose, UDP-[^{14}C]-glucose, or GDP[^{14}C]-mannose for one hour. The labeled neutral sugars in the products were analyzed as described in Materials and Methods. Label incorporated from UDP-[^{14}C]-galactose was recovered as [^{14}C]-galactose and [^{14}C]-mannose. Label from UDP-[^{14}C]-glucose was recovered as [^{14}C]-glucose, [^{14}C]-galactose, and [^{14}C]-mannose. Label from GDP-[^{14}C]-mannose was recovered as [^{14}C]-mannose, [^{14}C]-galactose, and [^{14}C]-glucose. These results in lymphocytes contrast earlier work with fibroblasts in which a lack of epimerization was presented as evidence that nucleotide sugars are not taken up intracellularly (Patt and Grimes, 1974).

Further support for epimerization of galactose was obtained by analyzing glycolipids labeled by the simultaneous incubation of Con A stimulated cells with UDP-[^{14}C]-galactose and [^{3}H]-galactose (Fig. 5). Glycolipids extracted from these cells were separated by thin layer chromatography. The free galactose must be taken up by the cell for conversion to UDP-galactose prior to incorporation and can be epimerized intracellularly. Thus label from [^{3}H]-galactose is incorporated into

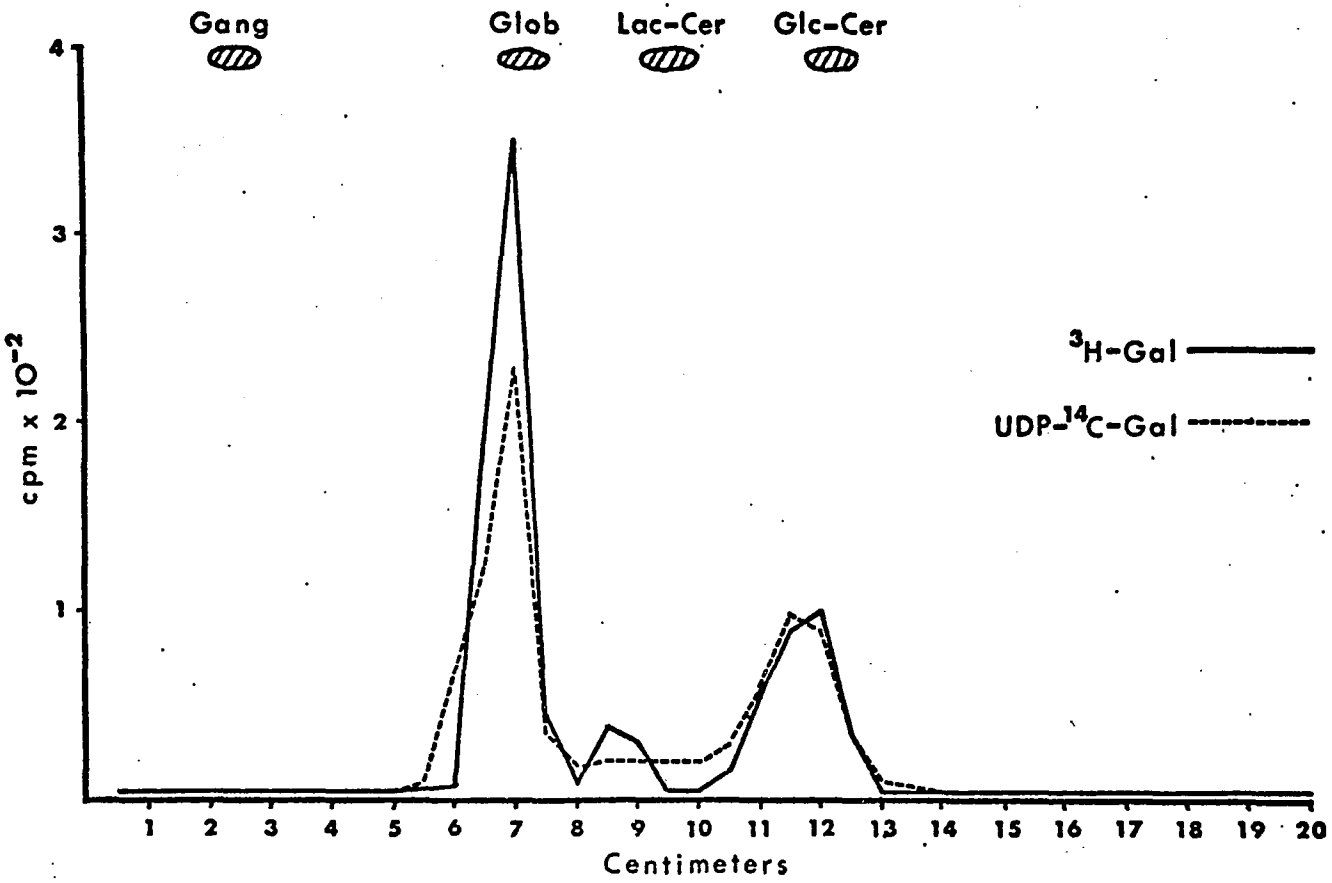


Fig. 5. Thin layer chromatography (TLC) of glycolipids from Con A stimulated lymphocytes incubated simultaneously with UDP-[^{14}C]-galactose and [^{3}H]-galactose.

After extraction of glycolipids, TLC was performed with precoated Silica Gel-G sheets (Brinkman Institute, Inc., Burlingame, Ca.) in a solvent of chloroform : methanol : 2.5 N NH_3 (60:35:8). The authentic markers indicated at the top are mono- and disialoganglioside migrating together (Gang), globoside (Glob), lactosylceramide (Lac-Cer), and glucosylceramide (Glc-Cer).

glucosylceramide as well as galactose-containing glycolipids. In contrast to results obtained with mouse fibroblasts (Patt and Grimes, 1974), glucosylceramide was also labeled by UDP-[^{14}C]-galactose. Thus, in lymphocytes, sugars from added nucleotide sugar substrates are exposed to the action of epimerases at some time before they are finally added to glycoproteins and glycolipids by glycosyltransferases. Further evidence in a later section supports the concept that this is not an indication of the cell's ability to transport the nucleotide sugar, but rather its ability to degrade the substrate, allowing transport of the breakdown products.

The Use of Adenosine-5'-Monophosphate to Prevent Substrate Hydrolysis

Adenosine-5'-monophosphate (5'-AMP) has been shown to be effective in inhibiting the action of pyrophosphatases in degrading nucleotide sugars (Bischoff, Wilkening, and Decker, 1973; Decker and Bischoff, 1972; and Geren and Edner, 1974). Con A stimulated spleen cells were therefore assayed for incorporation from both UDP-[^{14}C]-galactose and [^{3}H]-galactose either in the presence or in the absence of 5'-AMP. Metabolic alteration of both labeled molecules was determined by paper chromatography of TCA soluble material remaining after the one hour incubation period. The results are shown in Fig. 6. It can be seen that 5'AMP completely prevented degradation of UDP-[^{14}C]-galactose at the concentration used (5mM). The normal conversion of free galactose to galactose-1-phosphate and UDP-galactose was unaffected. A similar experiment demonstrated that 5'AMP was equally as effective in inhibiting the degradation of GDP-[^{14}C]-mannose by intact Con A stimulated cells (Fig. 7).

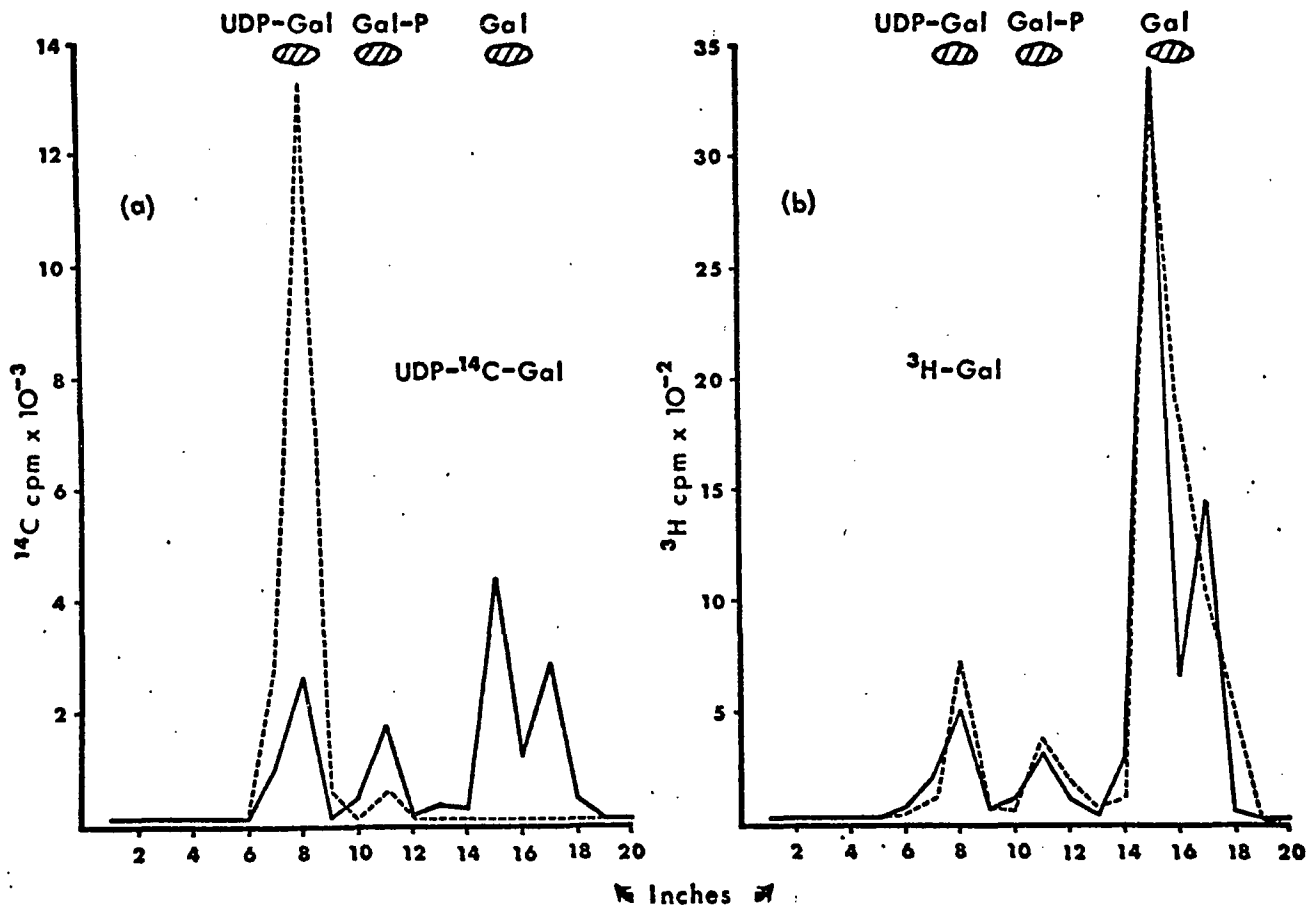


Fig. 6. Effects of 5'-AMP on metabolism of UDP-[^{14}C]-galactose and [^{3}H]-galactose by Con A stimulated lymphocytes.

Aliquots were incubated with both isotopes simultaneously in the presence (dashed line) and absence (solid line) of 5 mM 5'-AMP. After TCA precipitation, aliquots were pelleted and the supernates chromatographed as described in the legend to Fig. 4. The standards indicated at the top are UDP-galactose, galactose-1-phosphate and galactose.

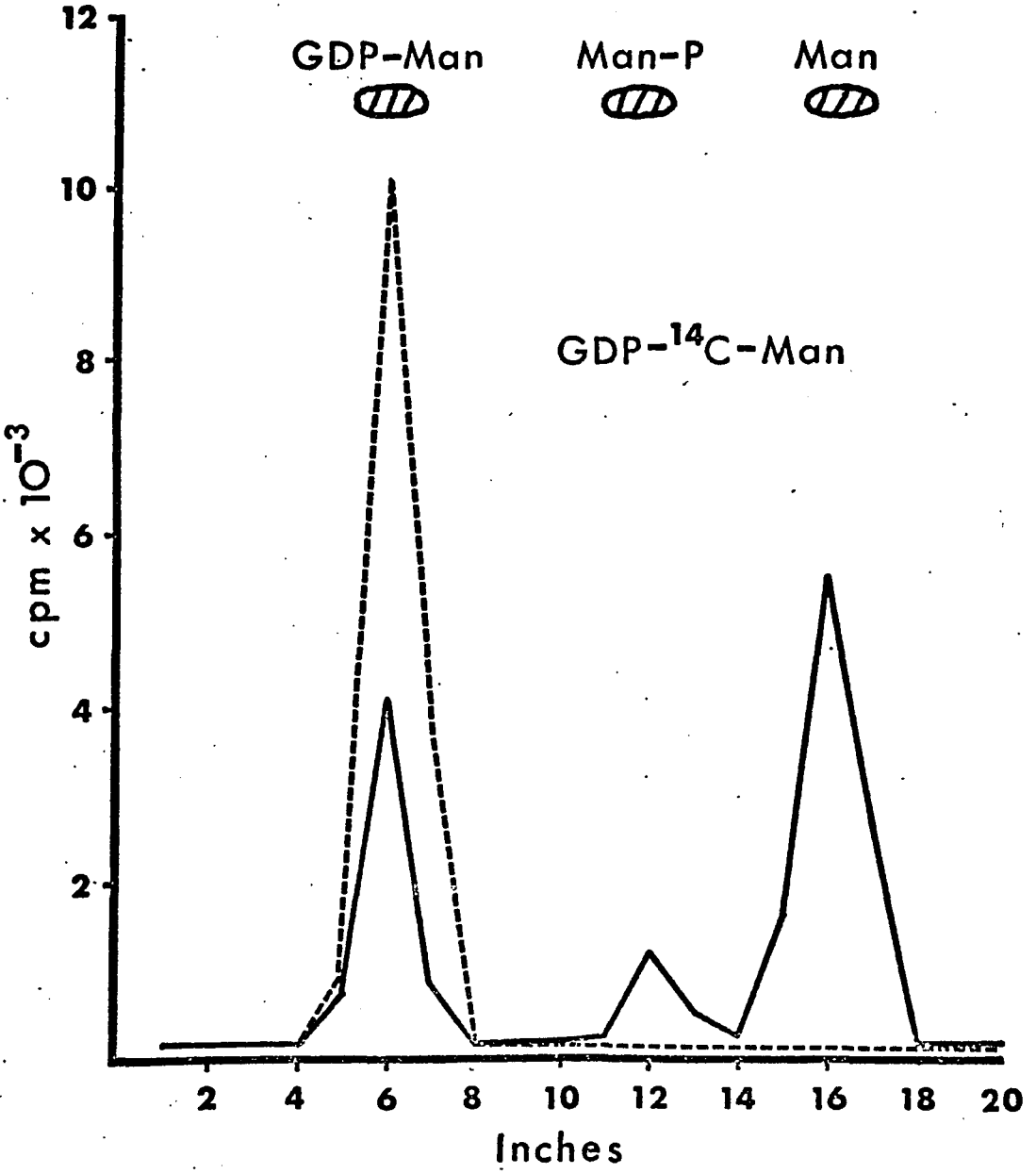


Fig. 7. Effects of 5'-AMP on breakdown of GDP-[^{14}C]-mannose by Con A stimulated lymphocytes.

The procedures used were identical to those described in the legend to Fig. 6. The standard markers are GDP-mannose, mannose-1-phosphate and mannose. The dashed line is obtained in the presence of 5 mM 5'-AMP and the solid line in its absence.

The Criteria for Determining an Ectoglycosyltransferase and Their Application to Murine Lymphocytes

Degradation of the substrate and intracellular incorporation of carbohydrates does not preclude the possibility of ectoglycosyltransferases. They may be present, but detection of their activity would be diminished by the rapid loss of substrate as well as masked by the higher level of intracellular incorporation.

Several criteria have been used to distinguish cell surface glycosyltransferase activity from intracellular activity (Patt and Grimes, 1974; and Deppert et al., 1974). These include addition of excess unlabeled sugars to inhibit transport, comparing activity of homogenates to that of intact cells, and using large molecular weight exogenous acceptors. To this list we must now add the criterion of preventing substrate degradation by the use of 5'AMP (see Patt et al., 1976).

Results obtained when Con A stimulated cells were incubated with both UDP-[^{14}C]-galactose and [^{3}H]-galactose in the presence of excess unlabeled UDP-galactose, galactose-1-phosphate, or galactose are shown in Fig. 8. Unlabeled UDP-galactose inhibited incorporation of label from the nucleotide sugar to a greater degree than from the free sugar. However, both galactose-1-phosphate and galactose inhibited incorporation from the nucleotide sugar and free sugar equally. The fact that inhibition with galactose-1-phosphate and galactose was similar supports the evidence presented earlier that the nucleotide sugar is degraded to the free sugar. The fact that incorporation from both UDP-[^{14}C]-galactose and [^{3}H]-galactose is inhibited equally indicates that UDP-galactose must be degraded to galactose before incorporation can occur. This implies

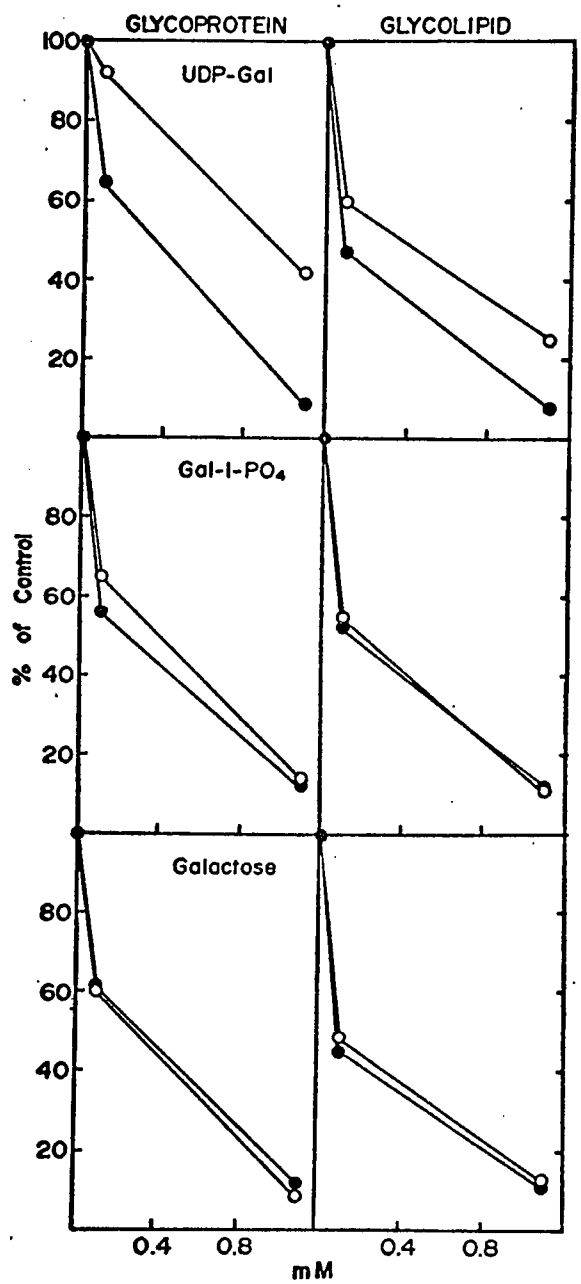


Fig. 8. Inhibition of labeling with UDP-[^{14}C]-galactose and [^{3}H]-galactose by unlabeled galactose derivatives.

Lymphocytes stimulated with Con A for 48 hr were incubated with both UDP-[^{14}C]-galactose (closed circles) and [^{3}H]-galactose (open circles) simultaneously. Two concentrations of UDP-galactose (top), galactose-1-phosphate (center), and galactose (bottom) were added. Incorporation into glycoproteins and glycolipids was measured in each sample. Each value is the mean of duplicate samples.

that galactosyltransferase activity is predominantly expressed as an intracellular activity in Con A stimulated lymphocytes.

Similar results were obtained with GDP-[^{14}C]-mannose (Fig. 9) and UDP-[^{14}C]-glucose (Fig. 10). Unlabeled GDP-mannose inhibited incorporation from GDP-[^{14}C]-mannose to a much greater extent than incorporation from [^{3}H]-mannose. (It should be noted, however, that quantitatively much less label was incorporated from [^{3}H]-mannose than from GDP-[^{14}C]-mannose. This is in contrast to nearly equal incorporation from UDP-[^{14}C]-galactose and [^{3}H]-galactose.) Unlabeled mannose inhibited incorporation from both GDP-[^{14}C]-mannose and [^{3}H]-mannose equally. Although low concentrations of unlabeled glucose increased incorporation from UDP-[^{14}C]-glucose, higher concentrations were again inhibitory.

In order to study directly the possibility of nucleotide sugar transport by Con A stimulated lymphocytes, cells were incubated with UDP-[^{14}C]-galactose and [^{3}H]-galactose in the presence of 5'-AMP. Radioactivity from both isotopes was then measured at successive times in three pools: the TCA precipitable material in the supernate, the TCA precipitable material associated with the cell pellet, and the TCA soluble material associated with the cell pellet (Fig. 11). The uptake of ^{3}H label from the free sugar into the intracellular acid soluble pool was rapid and did not reach equilibrium by 60 minutes. ^{14}C from the nucleotide sugar could be detected in this pool also, but the amount was very small, and did not continue to increase after 20 minutes.

The uptake of ^{3}H and ^{14}C can be more meaningfully compared by conversion of counts per minute to picomoles (pmol). These values are shown (in parentheses) in Fig. 11 for 60 minute points. It can be seen

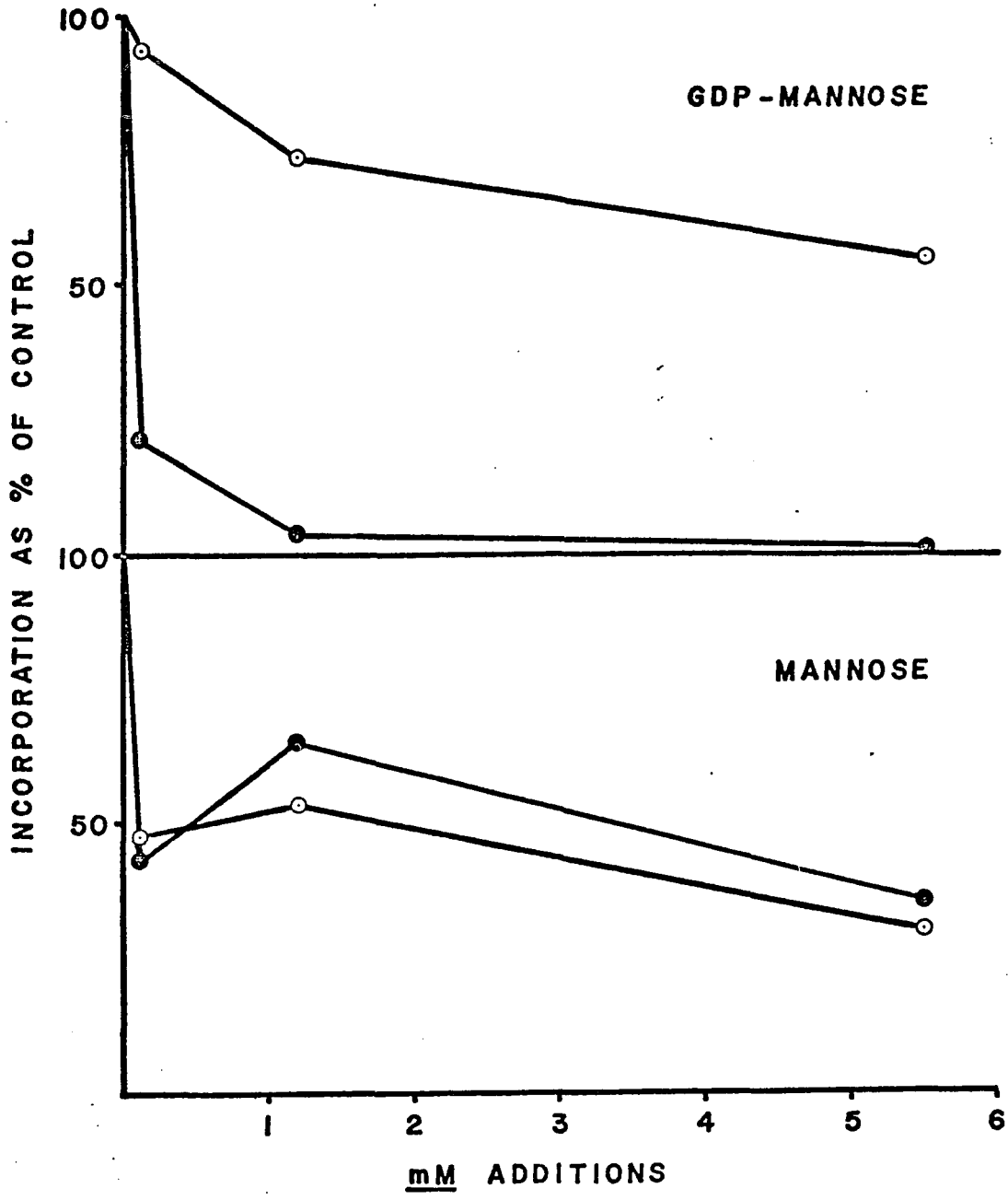


Fig. 9. Inhibition of labeling with GDP-[^{14}C]-mannose and [^{3}H]-mannose by unlabeled GDP-mannose and mannose.

Lymphocytes stimulated with Con A for 48 hr were incubated with both GDP-[^{14}C]-mannose (closed circles) and [^{3}H]-mannose (open circles) simultaneously. Three concentrations of GDP-mannose (top) and mannose (bottom) were added. Glycoproteins and glycolipids were not separated. Each value is the mean of duplicate samples.

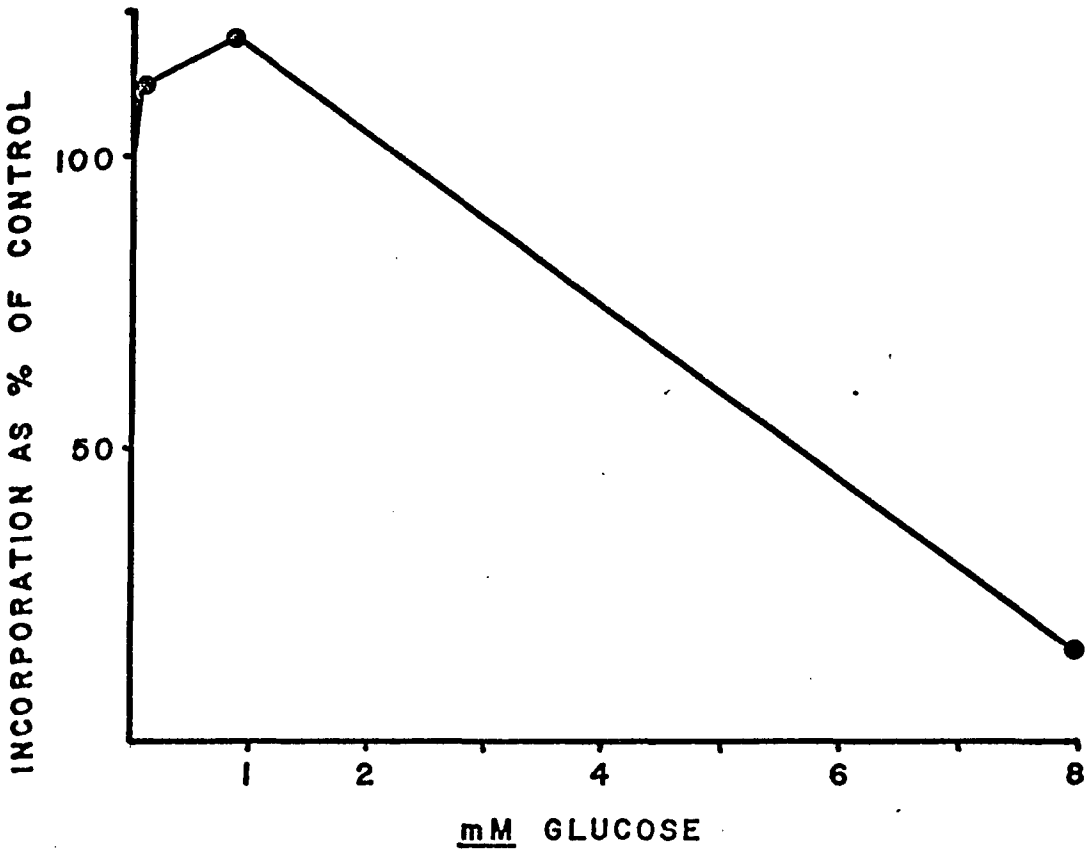


Fig. 10. Inhibition of labeling with UDP-[^{14}C]-glucose by unlabeled glucose.

Lymphocytes stimulated with Con A for 48 hr were incubated with UDP-[^{14}C]-glucose and three concentrations of unlabeled glucose. Each point is the mean of duplicate samples.

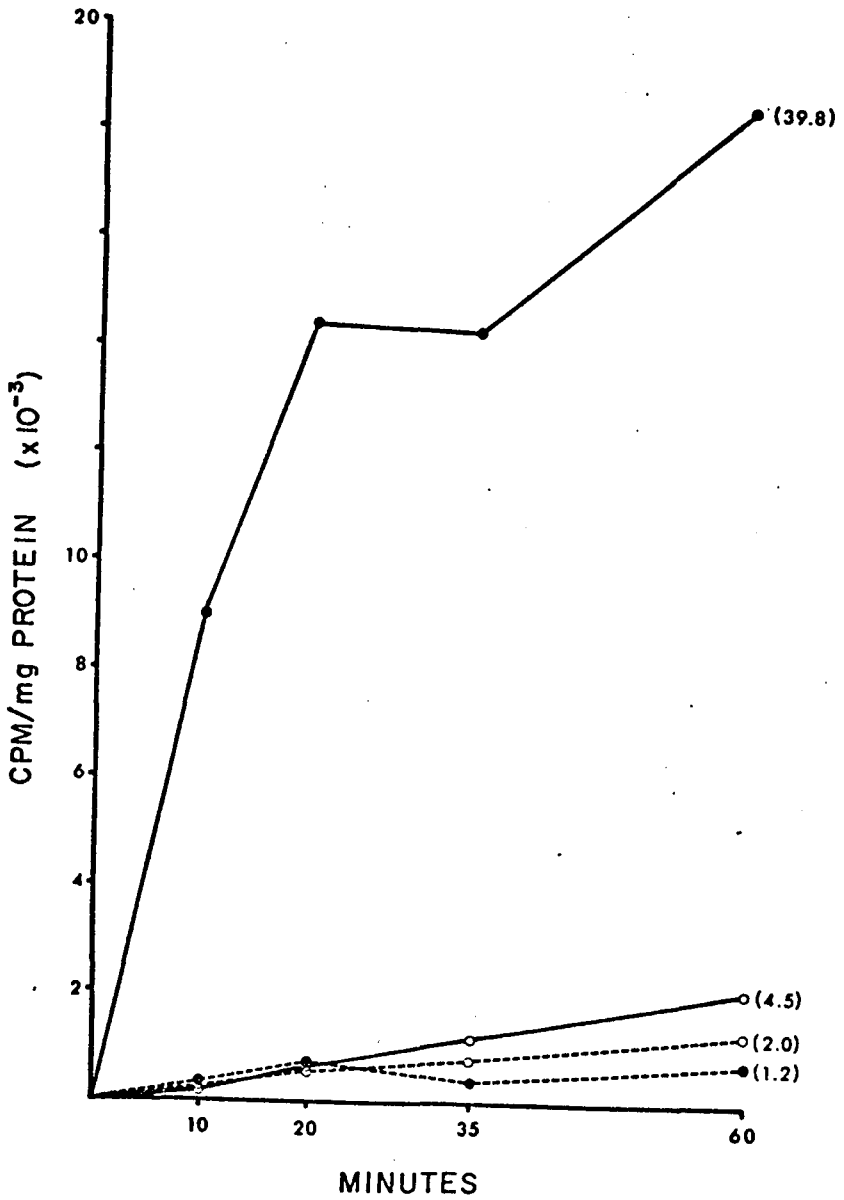


Fig. 11. Transport of UDP-[^{14}C]-galactose and [^{3}H]-galactose by Con A stimulated lymphocytes.

Lymphocytes were incubated with 5 mM, 5'-AMP, and both isotopes simultaneously. Reactions were stopped with the addition of 2 ml cold tris buffered saline, pelleted, and the supernates removed. Then 2 ml cold TCA was added to the cell pellets, and the precipitable material was separated from the soluble material by centrifugation and removal of the supernates. Specific activity of the [^{3}H]-galactose was 250 µCi/mmol, that for UDP-[^{14}C]-galactose was 274 µCi/mmol. Counting efficiency was 96% for ^{14}C and 46% for ^{3}H. 100 pmol (6 x 10^{4} dpm) of UDP-[^{14}C]-galactose and 180 pmol (1 x 10^{5} dpm) of [^{3}H]-galactose was used in each sample. Solid lines are for [^{3}H] cpm and dashed lines are for [^{14}C] cpm. Closed circles, TCA precipitable material; open circles, TCA soluble material.

that the ratio $^{3}H/^{14}C$ for a given sample is changed only slightly, and actually becomes greater.

As a new criterion for distinguishing cell surface glycosyltransferase activity, 5 mM 5'-AMP was added to assay mixtures and incorporation from both nucleotide sugars and free sugars measured. Contrasting results were obtained with UDP-[^{14}C]-galactose and GDP-[^{14}C]-mannose in Con A stimulated lymphocytes. Prevention of substrate hydrolysis by 5'-AMP reduced incorporation from UDP-[^{14}C]-galactose by 97%, whereas incorporation from [^{3}H]-galactose was only reduced by 35% (Fig. 12). Reduction of incorporation from the free sugar by 5'-AMP may be due to inhibition of a number of metabolic steps. There is some evidence that pyrimidine nucleotides can exert control over at least one glycosyltransferase (Bernaki, 1975). Purine nucleotides may also directly affect some glycosyltransferases.

5'-AMP did not reduce incorporation from either GDP-[^{14}C]-mannose or [^{3}H]-mannose (Fig. 13). The results shown in Fig. 12 suggest that very little galactosyltransferase is present as an ectoenzyme which can utilize extracellular nucleotide sugar directly. In addition, it appears that lymphocytes do not transport nucleotide sugars, in agreement with results from other mammalian systems (Bischoff et al., 1970; and Bischoff et al., 1973). The results shown in Fig. 13, on the other hand, indicate that Con A stimulated lymphocytes do possess an ectomannosyltransferase activity which is capable of utilizing extracellular substrate and endogenous acceptors. GDP-[^{14}C]-mannose need not be degraded to the free sugar for incorporation to occur. Why mannose inhibits incorporation from GDP-[^{14}C]-mannose (Fig. 9) is unknown. One explanation

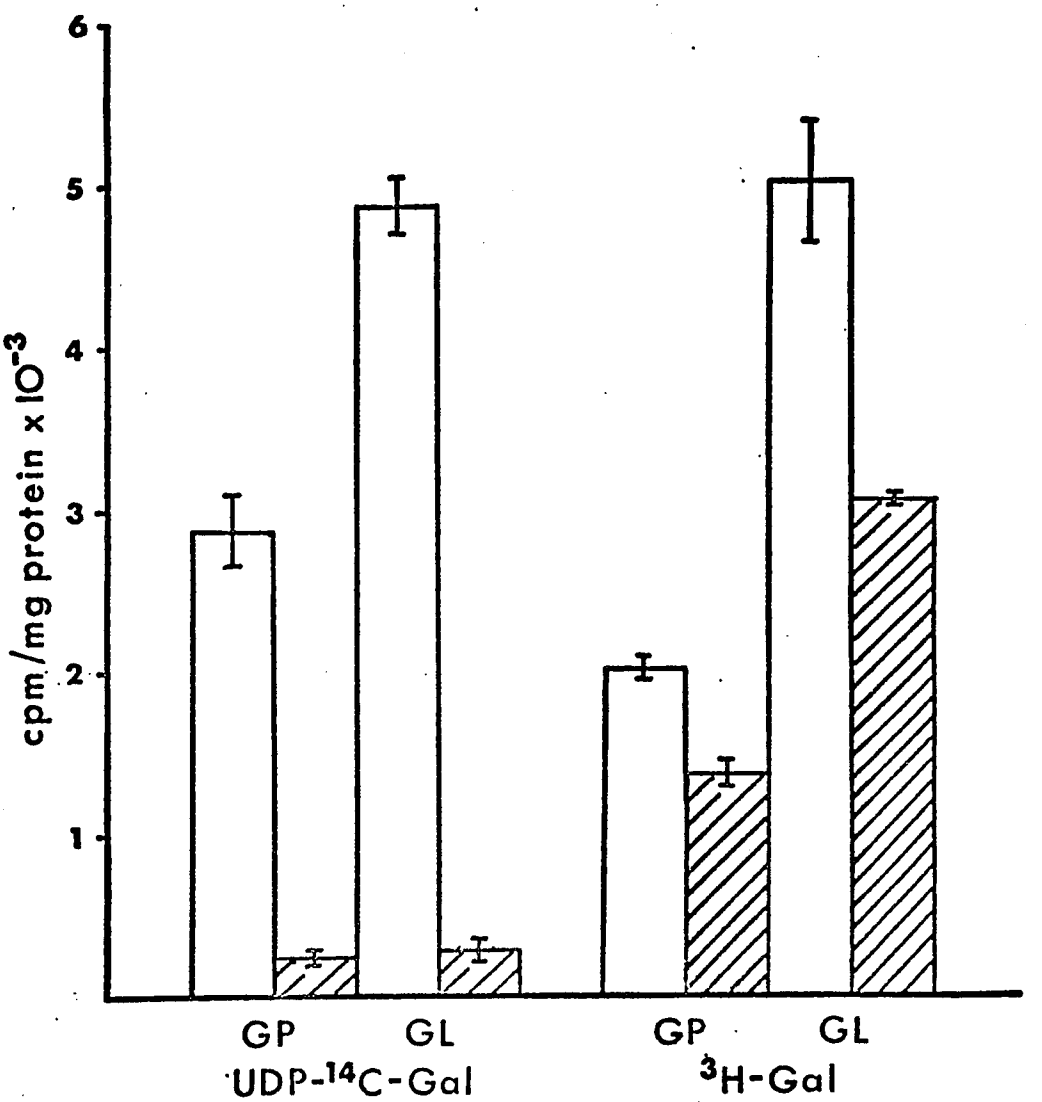


Fig. 12. Effects of 5'-AMP on incorporation of label from UDP-[^{14}C]-galactose and [^{3}H]-galactose by Con A stimulated lymphocytes.

Cells were assayed for incorporation of label into both glycoprotein and glycolipid from both isotopes simultaneously. Incubations were carried out in the presence (shaded bars) and absence (open bars) of 5 mM 5'-AMP. Each value is the mean of duplicate samples ± the range.

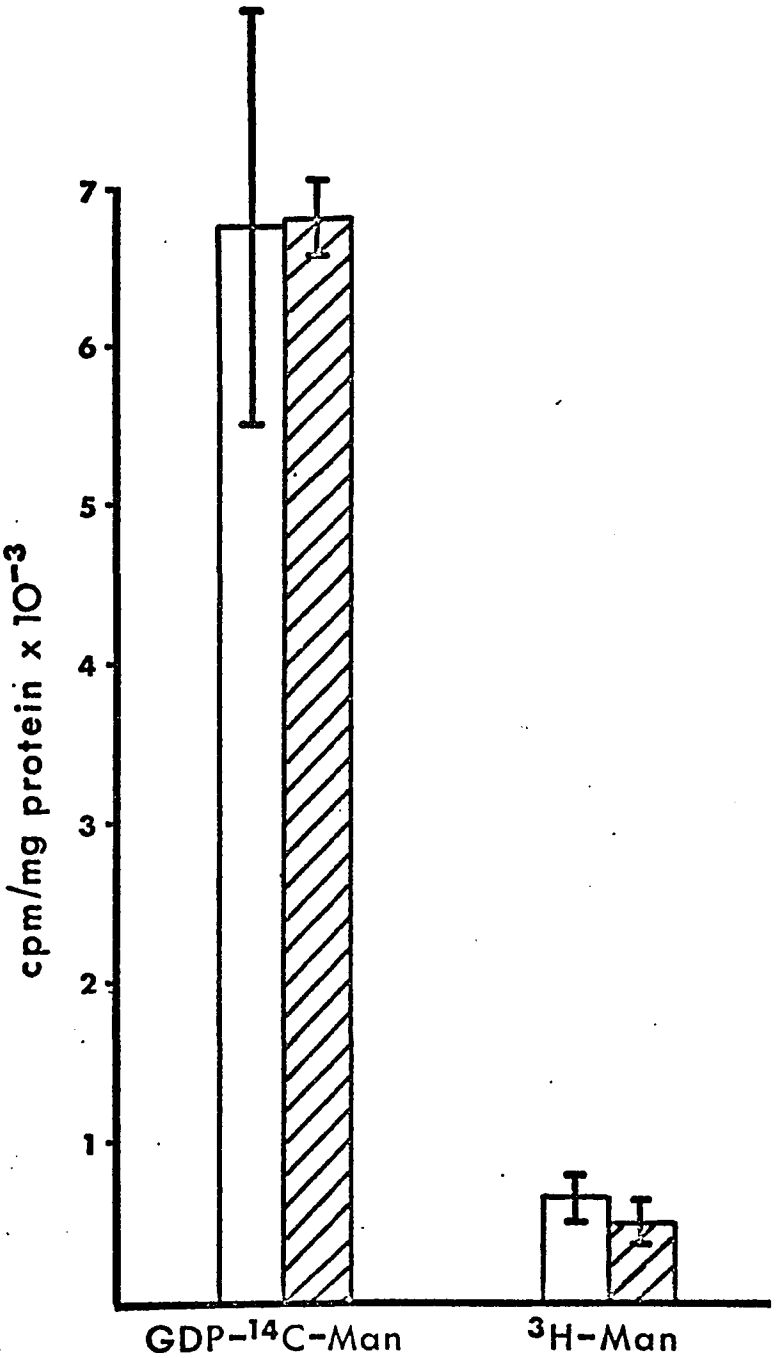


Fig. 13. Effects of 5'-AMP on incorporation of label from GDP-[^{14}C]-mannose and [^{3}H]-mannose by Con A stimulated lymphocytes.

Cells were assayed for total incorporation of label from both isotopes simultaneously. Incubations were carried out in the presence (shaded bars) and absence (open bars) of 5 mM 5'-AMP. Each value if the mean of duplicate samples ± the range.

could be that even though lymphocytes do not transport UDP-galactose readily (Fig. 11), they are able to transport GDP-mannose. Thus the labeled nucleotide sugar would be diluted with unlabeled nucleotide sugar in the intracellular pool by the addition of excess free mannose. Competition for the nucleotide sugar pool from free mannose is not in agreement, however, with the finding of low incorporation from [^{3}H]-l-mannose (Fig. 13).

To determine if the low level of incorporation in the presence of 5'-AMP was related to the time of assay, Con A stimulated spleen cells were assayed every 24 hr with or without 5'-AMP (Fig. 14). It can be seen that activity in the presence of 5'-AMP was low and constant throughout the culture period. Thus the residual activity is not stimulated in the incubations with Con A.

When galactosyltransferase activity associated with intact cells and with cells disrupted by Triton X-100 or freezing and thawing was compared (Table 2), it was found that the detergent decreased incorporation slightly, whereas freezing and thawing increased incorporation in the presence of 5'-AMP. Physically broken cells therefore allow incorporation by intracellular enzymes. Disruption with the detergent may interfere with enzyme-acceptor relationships, accounting for the slight decrease.

To test the possibility that ectogalactosyltransferase might be present on Con A stimulated spleen cells, but lack available acceptors, asialo-agalacto-fetuin was added to cell suspensions during the assay in the presence of 5'-AMP (Table 3). The exogenous acceptor decreased incorporation from the nucleotide sugar, but not the free sugar, into the

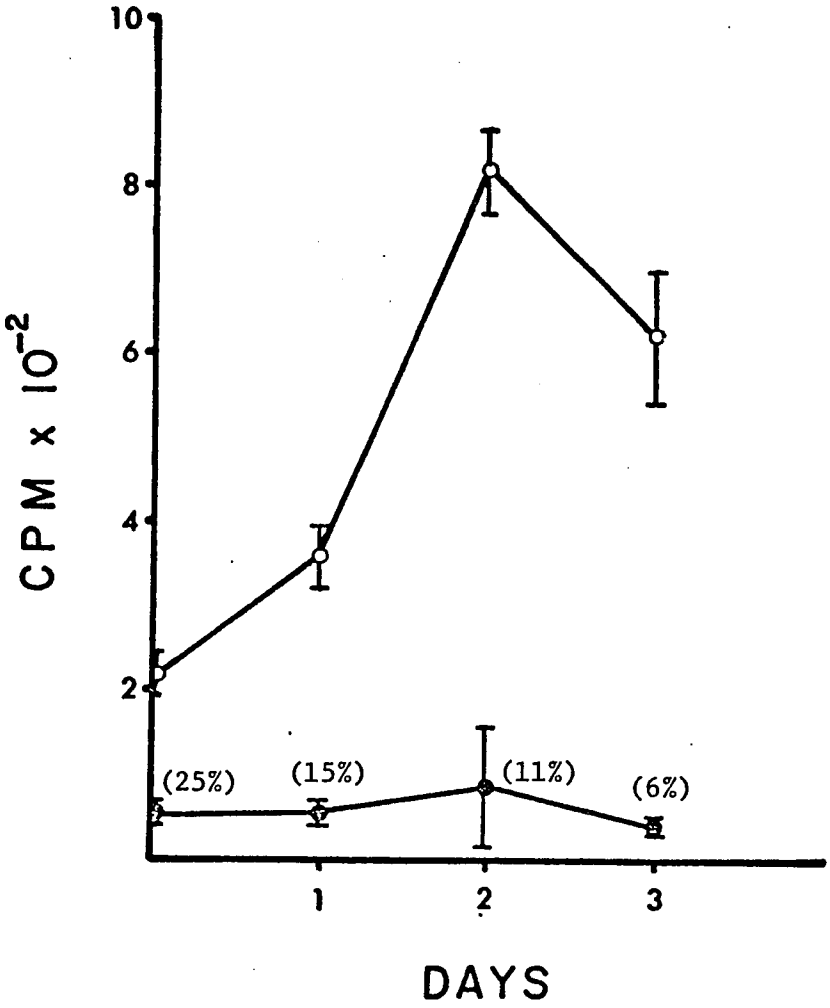


Fig. 14. Effect of 5'-AMP on the time course of incorporation of label from UDP-[^{14}C]-galactose by Con A stimulated lymphocytes.

Cells were assayed every 24 hr with (closed circles) and without (open circles) 5 mM 5'-AMP. The numbers in parentheses indicate incorporation in the presence of 5'-AMP as a percent of the activity observed in the absence of 5'-AMP. Each value is the mean of duplicate samples ± the range.

Table 2. Incorporation from UDP-[^{14}C]-galactose into glycoproteins and glycolipids by Con A stimulated spleen cell suspensions and homogenates.[a]

EXPERIMENT 1[b]	5'-AMP	^{14}C cpm/mg protein	
		Glycoprotein	Glycolipid
Cell Suspensions	-	4766 ± 30	825 ± 196
	+	2333 ± 230	575 ± 190
Triton X-100 Homogenates	-	3159 ± 120	804 ± 8
	+	1935 ± 110	387 ± 199
EXPERIMENT 2		Glycoprotein + Glycolipid (not separated)	
Cell Suspensions	+	911 ± 40	
Frozen-Thawed Cells	+	1654 ± 264	

a. In Experiment 1, identical aliquots of 48 hr Con A stimulated spleen cells were assayed in TBS, or TBS + 0.5% Triton X-100. In Experiment 2, identical aliquots were assayed normally, or after three rapid cycles of freezing and thawing.

b. It should be noted that incorporation by cell suspensions in Experiment 1 with 5'-AMP was very high, indicating the possibility of broken cells in the cell suspension.

Table 3. Incorporation from UDP-[^{14}C]-galactose and [^{3}H]-galactose into endogenous and exogenous acceptors[a] by Con A stimulated spleen cells, in the presence of 5'-AMP.

UDP-[^{14}C]-galactose	dpm/mg protein	
	Cell Pellet	Supernate
Cells alone	915 ± 63	52 ± 36
Cells plus asialo-agalacto-fetuin	187 ± 38	563 ± 58
[^{3}H]-galactose		
Cells alone	789 ± 30	122 ± 7
Cells plus asialo-agalacto-fetuin	848 ± 76	79 ± 30

a. The exogenous acceptor asialo-agalacto-fetuin was prepared as described in Materials and Methods. 250 µg in 25 µl was added to each assay tube. 25 µl of buffer was added to the tubes not receiving the acceptor. At the end of the 60 min incubation period, reactions were stopped with the addition of 2 ml cold buffer, vortexed, and pelleted at 150 g for 5 min. The supernate, containing the soluble acceptor, was transferred to a second tube, and 2 ml cold 10% TCA was added to both pellets and supernates. TCA insoluble material from each was then collected on glass fiber filter pads and radioactivity was measured. Each value is the mean of duplicate samples ± the range. When the acceptor was added to 100 ml of TBS without cells, radioactivity in the TCA precipitate was at the level of background.

cell pellet. TCA insoluble material in the supernates only evidenced incorporation from the nucleotide sugar. This would indicate that Con A stimulated lymphocytes do possess an ectogalactosyltransferase which can utilize extracellular, but not intracellular, UDP-galactose for transfer of galactose to an exogenous acceptor. The acceptor seems to interfere with incorporation from UDP-galactose, but not free galactose, into the cell itself. This is consistent with the interpretation that the incorporation observed is occurring at the cell surface. Another interpretation which cannot be excluded is that trypan blue positive cells transfer galactose to the exogenous acceptor at intracellular sites. They would be incapable of converting [^{3}H]-galactose to the nucleotide sugar due to a lack of free energy. Thus an ectogalactosyltransferase can only be reported as a possibility until methods are devised for cleanly removing nonviable cells.

When 5'-AMP was included in assays for incorporation from the other nucleotide sugars (Table 4) it decreased incorporation from UDP-[^{14}C]-glucose and CMP-[^{14}C]-N-acetylneuraminic acid, but actually increased incorporation from UDP-[^{14}C]-N-acetylglucosamine. This could be interpreted to mean that N-acetylglucosaminyl transferases and acceptors are available at the cell surface. In the absence of 5'-AMP, the nucleotide hexosamine is unavailable for use by the ectoglycosyltransferase, due to degradation by pyrophosphatases, and the charged free hexosamine is probably poorly transported.

Although incorporation from CMP-[^{14}C]-N-acetylneuraminic acid in the presence of 5'-AMP was low in Con A stimulated cells, it was found that treatment of the cells with Vibrio cholera neuraminidase before

Table 4. Effect of 5'-AMP on incorporation from UDP-[^{14}C]-glucose, UDP-[^{14}C]-N-acetylglucosamine and CMP-[^{14}C]-N-acetylneuraminic acid by Con A stimulated spleen cells.[a]

Substrate	^{14}C cpm/mg protein			
	Glycoprotein		Glycolipid	
	(-)	5'-AMP	(-)	5'-AMP
UDP-[^{14}C]-glucose	338 ± 105	190 ± 1	1065 ± 792	630 ± 354
UDP-[^{14}C]-N-acetylglucosamine	310 ± 14	681 ± 27	58 ± 3	137 ± 0
CMP-[^{14}C]-N-acetylneuraminic acid	99 ± 11	60 ± 27	5 ± 27	47 ± 3

a. Incorporation from each substrate was assayed after 48 hr in culture with Con A. Assays were done with or without 5 mM 5'-AMP. Each value is the mean of duplicate samples ± the range.

assay enhanced the incorporation (Fig. 15). The degree of enhancement was dependent on the concentration of enzyme used. This procedure was assumed to generate sialyl acceptors at the cell surface, but it cannot be assumed that the increased sialyltransferase activity was on the cell surface because the assay was not performed with added 5'-AMP.

Ectoglycosyltransferases on Other Cells

Red Blood Cells

To test whether contaminating red blood cells contributed to the observed incorporation from nucleotide sugars, spleen cell suspensions were treated with 0.83% NH_4Cl before culture with Con A. This procedure lysed essentially all red cells and reduced the mononuclear cell yield by 30%. After 48 hr in culture, incorporation of both [^{3}H]-thymidine and CMP-[^{14}C]-N-acetylneuraminic acid were increased twofold. Fresh murine peripheral blood erythrocytes, either normal or treated with Vibrio cholera neuraminidase failed to incorporate CMP-[^{14}C]-N-acetylneuraminic acid. At least for sialic acid then, red blood cells do not contribute to the incorporation of carbohydrate from nucleotide sugars in spleen cell suspensions.

B Cells

Murine lymphocytes have been classified into two major classes, based on surface markers and function. These are the thymus derived, or T cells and bone marrow derived, or B cells. Soluble Con A is known to stimulate only T cells in the mouse. Endotoxin, or lipopolysaccharide (LPS) stimulates only B cells (Greaves and Janossy, 1972). In Fig. 16

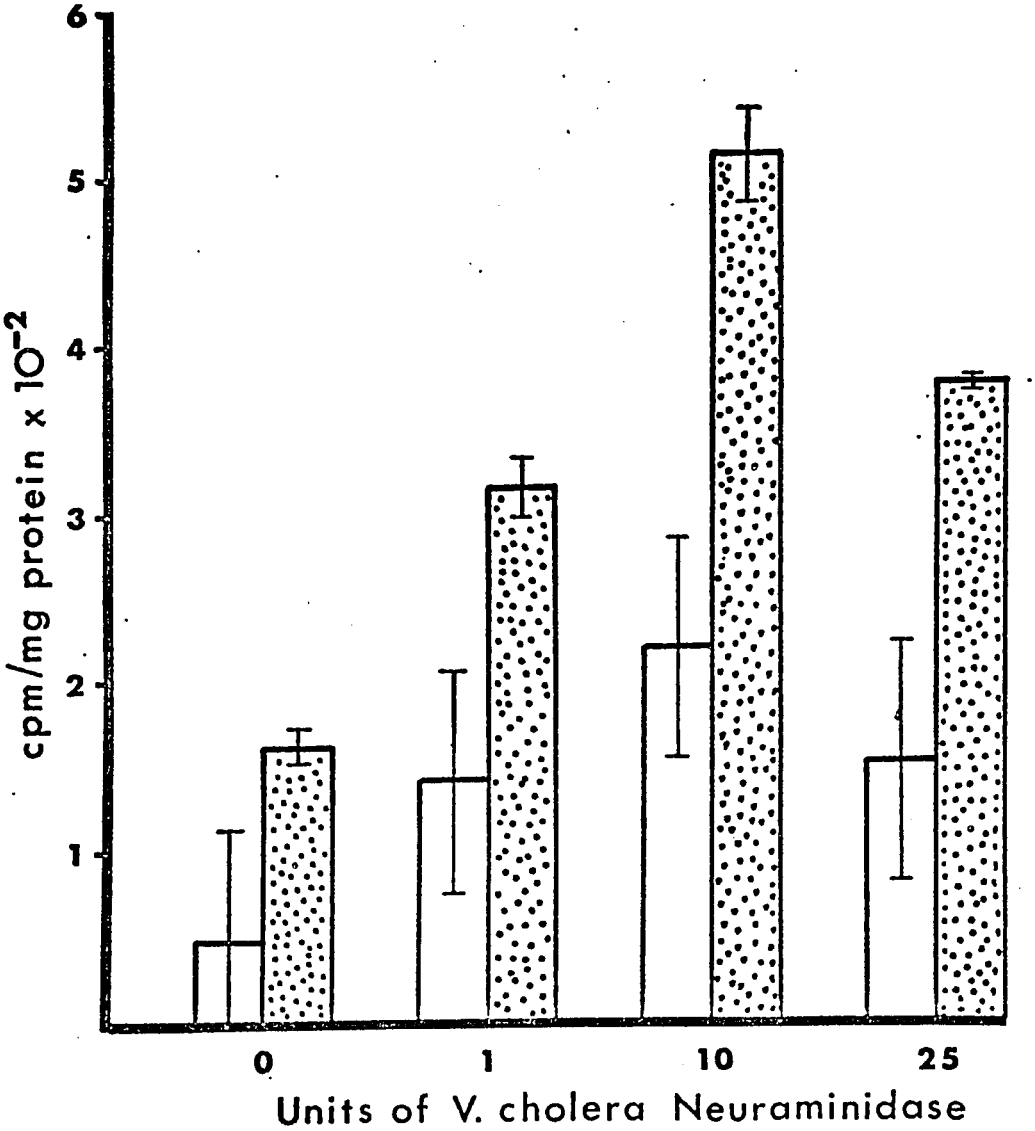


Fig. 15. Incorporation of label from CMP-[^{14}C]-N-acetylneuraminic acid by Con A stimulated lymphocytes after treatment with Vibrio cholera neuraminidase.

Cells were incubated with the enzyme in phosphate suffered saline (pH 7.4), for 30 min at 37°C, washed three times, and assayed for incorporation into glycoprotein only. Each value is the mean of duplicate samples $\pm$ the range.

it can be seen that DNA synthesis during incubation of spleen cells with LPS reached a peak at two days. At this time, LPS stimulated cultures contained 25% blast cells, while control cultures contained 3%. Viability was 65% in both. Incorporation of radioactive carbohydrate from several labeled nucleotide sugars in the presence of 5'-AMP was low, and increased only slightly with three of the five substrates (Fig. 16). In the absence of 5'-AMP, incorporation from UDP-[^{14}C]-galactose was increased sixfold. Comparing these results to those obtained with Con A stimulated T cells, a major difference is apparent. Whereas Con A stimulated T cells incorporate much more label from GDP-[^{14}C]-mannose than from UDP-[^{14}C]-galactose in the presence of 5'-AMP, LPS stimulated B cells incorporate equivalent (albeit low) amounts of label from both substrates.

Lymph Node Cells and Thymocytes

Stimulation by Con A was also investigated in lymphoid cells from other anatomical sources -- the lymph nodes and thymus. These cell populations differ from spleen cells in that very few erythrocytes are present and the T cell/B cell ratios are different. Whereas the spleen contains a majority of B cells, lymph nodes contain a majority of T cells. Thymuses contain almost exclusively T cells, although they are for the most part immunologically immature. Con A usually stimulates greater [^{3}H]-thymidine incorporation in lymph node cells, and lesser incorporation in thymocytes as compared to spleen cells.

Con A stimulation of carbohydrate incorporation from UDP-[^{14}C]-galactose in the presence of 5'-AMP in lymph node cells and thymocytes

Fig. 16. Time course of DNA synthesis and incorporation of labeled carbohydrate from nucleotide sugar substrates at 48 hr by LPS stimulated mouse spleen cells.

Top: Time course of DNA synthesis in spleen cells incubated with 50 μg/ml LPS (closed circles) and in control cells (open circles). DNA synthesis was assayed every 24 hr as described in the legend to Fig. 1. Each value is the mean of duplicate samples from duplicate tubes. Bottom: Incorporation of labeled carbohydrate from nucleotide sugar substrates by spleen cells incubated with or without 50 μg/ml LPS for 48 hr. 5 mM 5'-AMP was included in all assays except for those represented by the pair of bars on the far left (shaded), which are from a separate experiment. Glycoproteins and glycolipids were not separated. Each value is the mean of duplicate samples ± the range.

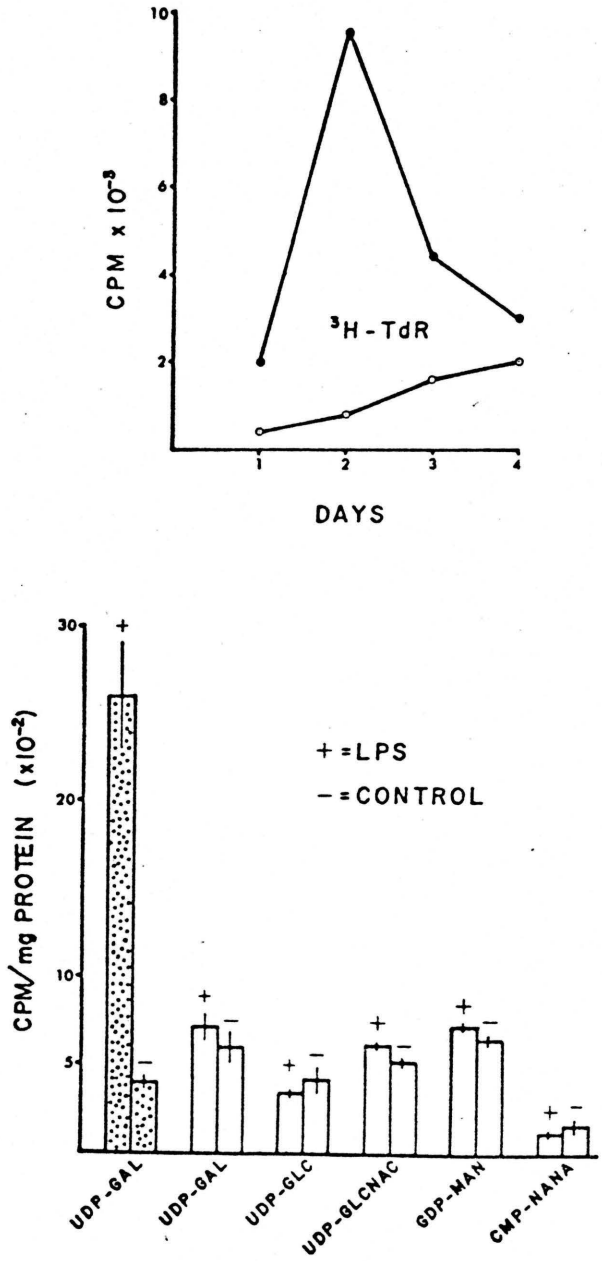


Fig. 16. Time course of DNA synthesis and incorporation of labeled carbohydrate from nucleotide sugar substrates at 48 hr by LPS stimulated mouse spleen cells.

is shown in Fig. 17. Incorporation by lymph node cells is much greater than that by spleen cells. The incorporation is qualitatively different for both lymph node cells and thymocytes in that very little activity is seen in the glycolipid fractions as compared to the glycoprotein fractions. Total incorporation with 5'-AMP was 70-95% of that found without 5'-AMP in thymocytes. The proportion was 25% in lymph node cells. In several experiments, the proportion was never higher than 15% in spleen cells at 48 hr.

Incorporation from four other nucleotide sugar substrates was tested in thymocytes and found to be undetectable or very low and variable, and not stimulated by Con A. These were UDP-[^{14}C]-glucose, UDP-[^{14}C]-N-acetylglucosamine, GDP-[^{14}C]-mannose, and CMP-[^{14}C]-N-acetyl-neuraminic acid.

These results demonstrate that cells from different lymphoid organs express different surface activities upon stimulation by Con A. This suggests several possibilities: (1) Con A might be stimulating different subpopulations of T cells from different organs, (2) different cellular interactions might occur, causing the cells to express surface activity differently, or (3) different levels of maturation in the three organs might vary the responsiveness to Con A.

Macrophages

In several tests of galactosyltransferase activity associated with intact purified macrophages, and in the presence of 5'-AMP, widely varying values were obtained. This could not be correlated with the varying number of cells used in each assay, but did follow a trend

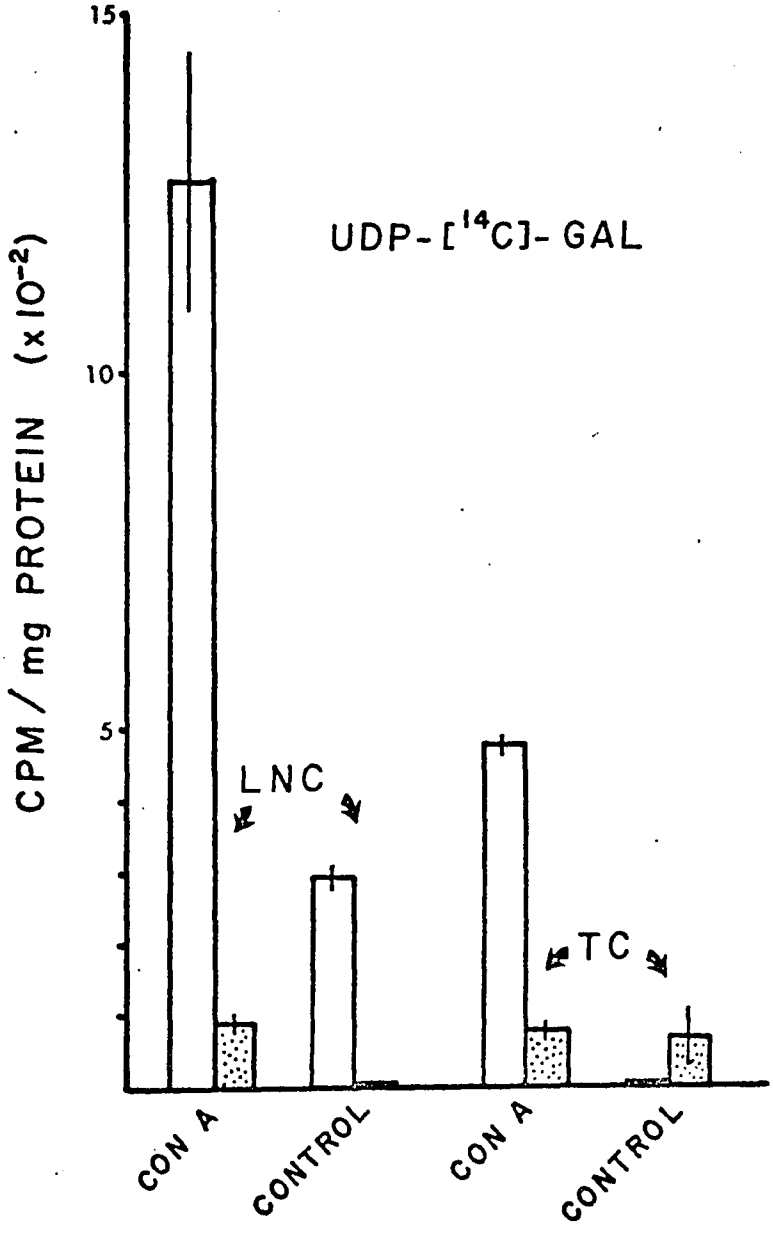


Fig. 17. Incorporation of label from UDP-[^{14}C]-galactose in the presence of 5'-AMP by murine lymph node cells and thymocytes.

Cultures were set up as described for spleen cells at 5 x 10^6 cells per ml, and incubated with or without 10 µg/ml Con A for 48 hr. Incorporation was measured into both glycoproteins (open bars) and glycolipids (shaded bars) in the presence of 5 mM 5'-AMP. Each value is the mean of duplicate samples ± the range.

related to the length of time the cells were cultured before assay (Table 5). Macrophages obtained by intraperitoneal injection of thioglycollate broth were initially in an 'activated' state. Numerous cytoplasmic vacuoles could be seen and phagocytic activity was high. As they sat in culture, either on glass or on collagen as described in Materials and Methods, they attached and spread out, forming a monolayer. Gradually the cytoplasmic vacuoles disappeared and the cells assumed a fibroblast-like morphology. These changes may be related to the diminishing ectogalactosyltransferase activity between two and five days.

To distinguish whether the concentration of 5'-AMP used was sufficient for use with purified macrophages, incorporation from UDP-[^{14}C]-galactose was assayed using increasing concentrations of 5'-AMP. Fig. 18 shows that the effect is actually maximal at a concentration of 1 mM and equal to the effect observed at 5 mM. This is similar to the dose-response relationship seen with Con A stimulated spleen cells and 3T3 fibroblasts. Thus the dose of 5'-AMP was not limiting and could not account for the variability of incorporation seen with macrophages.

When other nucleotide sugar substrates were tested with purified macrophages in the presence of 5'-AMP (Fig. 19), the macrophages had been cultured for five days, and incorporation from UDP-[^{14}C]-galactose was low, as seen in Table 5. However, a higher level of activity was still present for the substrates UDP-[^{14}C]-glucose, UDP-[^{14}C]-N-acetyl-glucosamine and GDP-[^{14}C]-mannose. Incorporation from CMP-[^{14}C]-N-acetylneuraminic acid was undetectable, unless the cells were pretreated with neuraminidase. The major difference noted between macrophages and Con A stimulated spleen cells is the high level of incorporation from

Table 5. Incorporation from UDP-[^{14}C]-galactose by macrophages; possible relationship between length of time in culture and incorporation observed.[a]

Time in culture before assay	With 5'-AMP		Without 5'-AMP	
	GP[b]	GL[c]	GP	GL
Two days	1621 ± 34	751 ± 164	8107 ± 785	2356 ± 537
Four days	684 ± 4	113 ± 58	ND[d]	ND
Four days	358 ± 20	51 ± 20	782 ± 37	355 ± 44
Five days	200 ± 377	ND	1889 ± 66	ND

a. Mouse peritoneal exudate cells were collected and cultured on collagen layers as described in Materials and Methods for the periods at the left. At the time of assay, macrophages were released with collagenase, washed, and assayed for incorporation with and without 5 mM 5'-AMP. The four rows of data represent four separate experiments. Each value is the mean of duplicate samples ± the range.

b. glycoprotein

b. glycolipid

d. not done

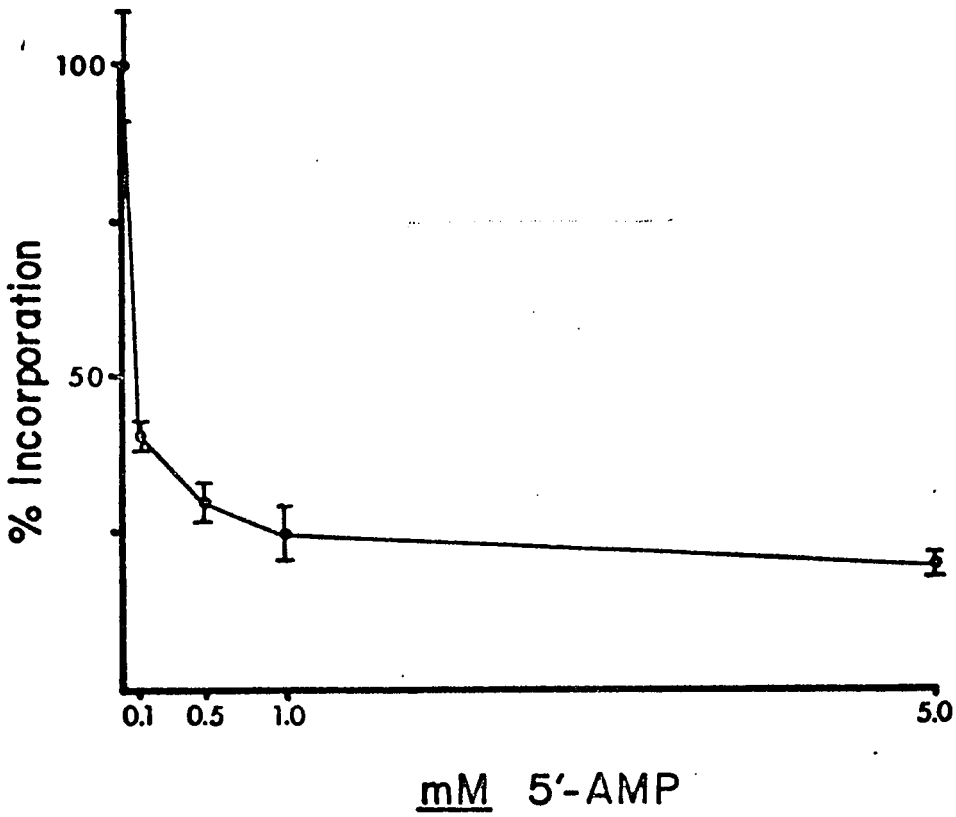


Fig. 18. Effect of concentration of 5'-AMP on incorporation from UDP-[^{14}C]-galactose by macrophages.

Purified macrophages were obtained as described in Materials and Methods. After two days in culture on collagen gel layers, the cells were removed with collagenase and assayed as described for spleen cells with four dilutions of 5'-AMP such that the final concentrations were as indicated on the abscissa. Each value is the mean of duplicate samples $\pm$ the range.

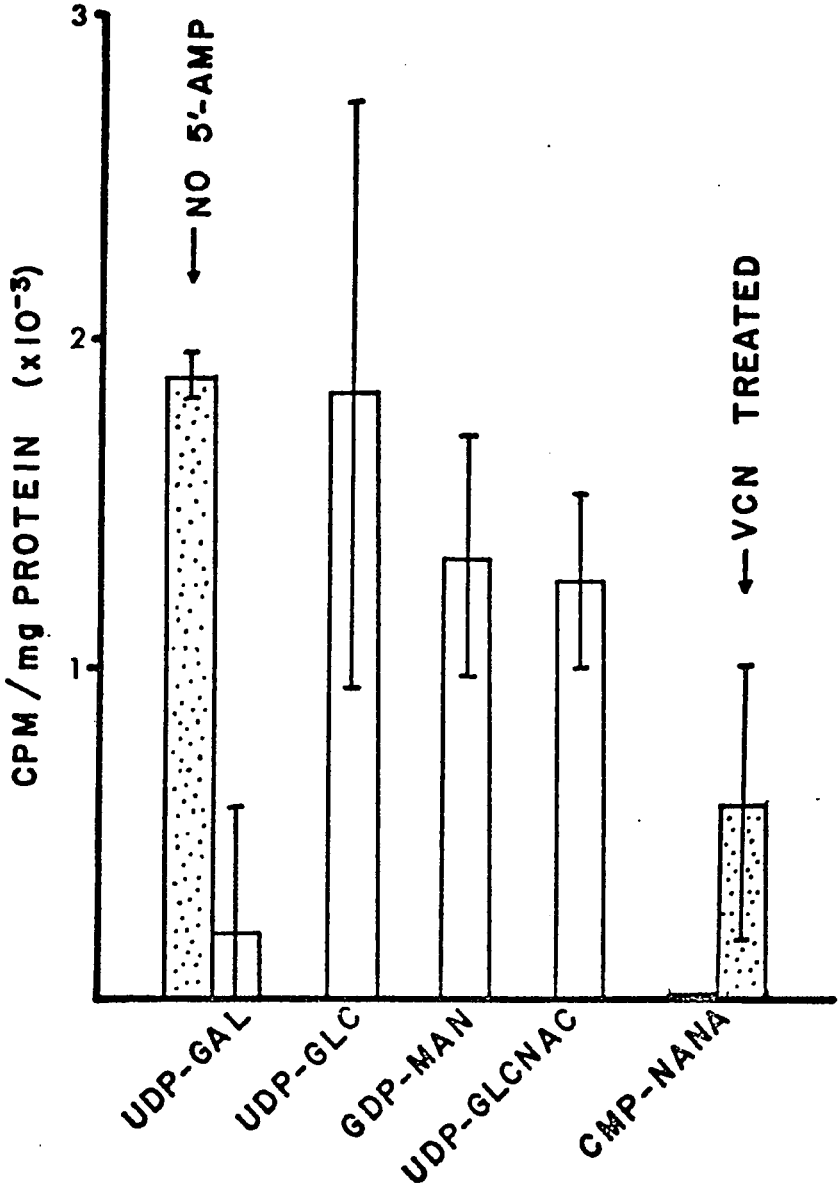


Fig. 19. Incorporation of labeled carbohydrate from nucleotide sugar substrates in the presence of 5'-AMP by purified macrophages.

Macrophages were obtained as described in Materials and Methods. After 4 days in culture on collagen gel layers, the cells were removed with collagenase and assayed with 5 mM 5'-AMP. Glycoproteins and glycolipids were not separated. On the far left, the shaded bar represents incorporation observed in the absence of 5'-AMP. On the far right, the shaded bar represents incorporation observed after pretreatment of the cells with 10 U/ml Vibrio cholera neuraminidase for 30 min at 37°C. Each value is the mean of duplicate samples ± the range.

UDP-[^{14}C]-N-acetylglucosamine, relative to the other substrates, in macrophages.

Disruption of macrophage integrity with Triton X-100 resulted in a slight increase in incorporation from UDP-[^{14}C]-galactose in the presence of 5'-AMP (Table 6). This contrasts with the results seen for lymphoblasts and may reflect a much higher level of intracellular activity in macrophages.

Induction of Glycoprotein and Glycolipid Synthesis With Other Lectins and Mitogens

Phytohemagglutinin (PHA) is thought to stimulate a subset of the cells which can be stimulated by Con A in the mouse. PHA binds preferentially to N-acetylgalactosamine residues on cell surfaces, whereas Con A binds preferentially to mannose residues. To compare the ability of each to stimulate incorporation from nucleotide sugars, mouse spleen cells were incubated with PHA for 48 hr, a time at which maximum DNA synthesis occurs. At that time, the cells were assayed for incorporation of several nucleotide sugars into glycoprotein and glycolipid, without 5'-AMP (Fig. 20). By comparing Fig. 20 to Fig. 3, it can be seen that PHA stimulates a pattern of incorporation similar to that stimulated by Con A. Since 5'-AMP was not included in the assays, these results are only an indication of overall complex carbohydrates synthesis, not necessarily taking place on the cell surface.

To compare the effects on spleen cells of other lectins, cells were incubated with either wheat germ agglutinin (WGA) which binds preferentially to N-acetylglucosamine, a lectin isolated from the mushroom Agaricus bisporus (AbL) which binds preferentially to gal-β(1→3)-galNAc,

Table 6. Incorporation from UDP-[^{14}C]-galactose by macrophage suspensions and homogenates.[a]

	5'-AMP	^{14}C cpm/mg protein	
		Glycoprotein	Glycolipid
Macrophage suspensions	-	782 ± 37	355 ± 44
	+	358 ± 20	51 ± 20
Triton X-100 homogenates	-	613 ± 183	67 ± 66
	+	419 ± 86	113 ± 5

a. Murine macrophages, cultured on collagen layers for four days as described in Materials and Methods, were assayed as whole cell suspensions or mixed with Triton X-100 (final concentration 0.5%). Each value is the mean of duplicate samples ± the range.

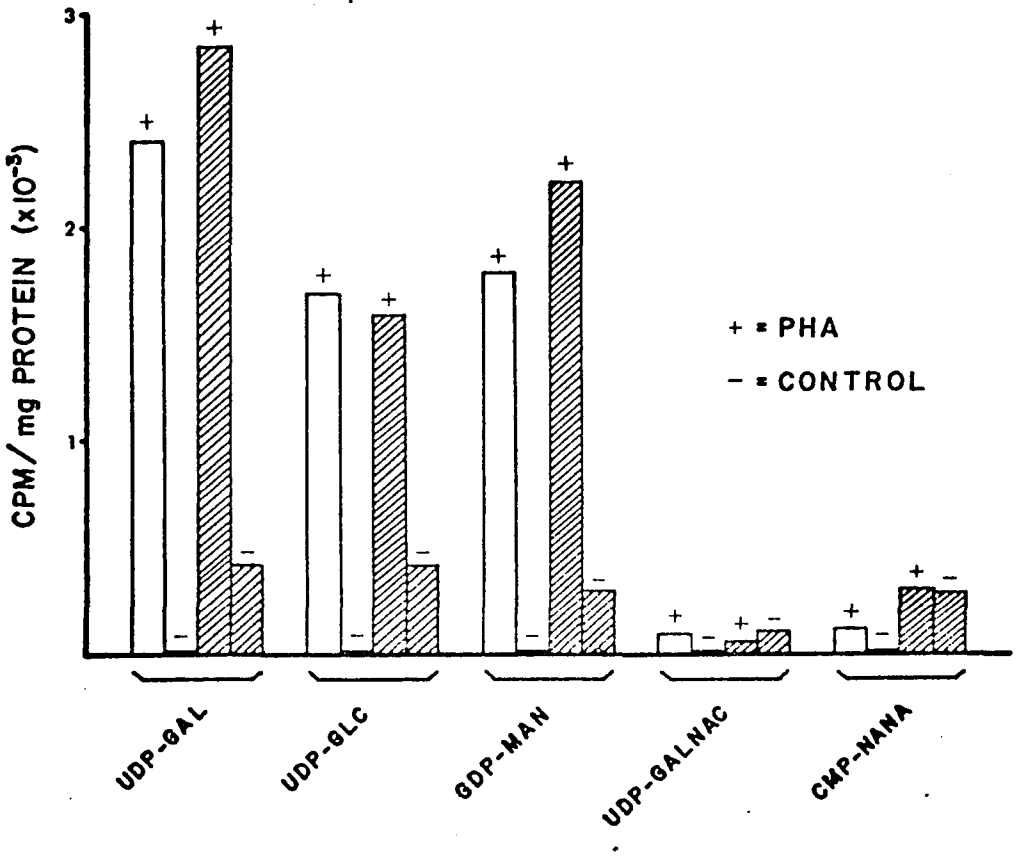


Fig. 20. Incorporation of labeled carbohydrate from nucleotide sugar substrates by mouse spleen cells incubated for 48 hr with PHA.

Assays were carried out without 5'-AMP. Incorporation was measured into both glycoproteins (open bars) and glycolipids (shaded bars). Each value is the mean of duplicate samples ± the range.

or Con A. At both 24 and 48 hr blastogenesis was assessed by both [^{3}H]-thymidine incorporation and actual counting of blast cells. Incorporation from UDP-[^{14}C]-galactose was measured into glycoprotein and glycolipid in the presence and absence of 5'-AMP (Table 7).

All three of the lectins caused agglutination to occur, but not all induced blastogenesis. Con A and AbL did, but WGA did not. AbL induced approximately the same percentage of microscopically identifiable blast cells as Con A did, but stimulated much less DNA synthesis. The level of incorporation from UDP-[^{14}C]-galactose induced by AbL was higher than that seen in control cells, but was less than that induced by Con A, similar to the relative amounts of DNA synthesis observed. Although WGA did not induce blastogenesis, it did increase incorporation from the nucleotide sugar and this incorporation was not decreased by addition of 5'-AMP. Control cells seem to have been slightly stimulated in this experiment, as both the percent blast cells and incorporation of carbohydrate were higher at 48 hr than at 24 hr. This may have been due to a serum effect.

In a similar experiment, thymocytes were incubated with WGA, AbL, LPS, or Con A for 48 hr (Table 8). All the carbohydrate-binding lectins (WGA, AbL and Con A) caused agglutination of the thymocytes, but the mitogen LPS did not. Only Con A induced blastogenesis in these cells, although DNA synthesis was increased slightly in cells incubated with AbL. All the substances increased incorporation from UDP-[^{14}C]-galactose compared to control cells. Incorporation in AbL cultures was nearly as great as that in Con A cultures. These assays were all done in the

Table 7. Incorporation from UDP-[^{14}C]-galactose by mouse spleen cells incubated with different lectins.[a]

Lectin	24 hr				48 hr					
			^{14}C cpm/mg protein				^{14}C cpm/mg protein			
							glycoprotein		glycolipid	
	SI[b]	%B[c]	glyco-protein	glyco-lipid	SI	%B	(-)	5'-AMP	(-)	5'-AMP
WGA	0.5	7	147 ± 7	(-)[d]	2.1	16	1027 ± 64	1709 ± 118	1250 ± 105	768 ± 114
AbL	2.9	18	111 ± 3	(-)	6.5	27	1515 ± 185	730 ± 100	610 ± 320	305 ± 185
Con A	22.2	18	490 ± 16	622 ± 174	34.7	53	5258 ± 464	580 ± 68	6812 ± 160	256 ± 39
Control	1.0	6	90 ± 5	(-)	1.0	17	841 ± 131	ND[e]	224 ± 168	ND

a. Procedures for counting blast cells and measuring incorporation of [^{3}H]-thymidine and UDP-[^{14}C]-galactose were as described in Materials and Methods. All cultures were at a cell density of 5 x 10^{6} cells/ml. The concentrations of the lectins were: WGA, 42 µg/ml; AbL, 35 µg/ml; and Con A, 10 µg/ml. Assays done with 5'-AMP used it at 5 mM. Each value for incorporation from UDP-[^{14}C]-galactose is the mean of duplicate samples ± the range.

b. Stimulation Index. Thymidine incorporation in stimulated cultures divided by that in control cultures.

c. Percent blast cells counted at the time of assay.

d. No incorporation into this fraction.

e. Not done.

Table 8. Incorporation from UDP-[^{14}C]-galactose by mouse thymocytes incubated with different lectins and LPS for 48 hr.[a]

(Glycoproteins and glycolipids not separated).

Addition	SI[b]	%B[c]	^{14}C cpm/mg protein
WGA	1.1	2	916 ± 402
AbL	1.6	2	3091 ± 136
Con A	16.2	9	4120 ± 228
LPS	1.2	3	1289 ± 632
Control	1.0	3	135 ± 246

a. Thymocytes were cultured at 5 x 10^6 cells/ml for 48 hr with each of the additions shown on the left. Procedures for counting blast cells, and measuring incorporation of [^{3}H]-thymidine and UDP-[^{14}C]-galactose were as described in Materials and Methods. Concentrations of the additions were: WGA, 48 µg/ml; AbL, 35 µg/ml; Con A, 10 µg/ml; and LPS, 50 µg/ml. Each value for incorporation of carbohydrate is the mean of duplicate samples ± the range.

b. Stimulation Index. Thymidine incorporation in stimulated cultures divided by that in control cultures.

c. Percent blast cells counted at the time of assay.

absence of 5'-AMP and are therefore only a measure of overall complex carbohydrate synthesis.

The Effects of Hydroxyurea on Con A Stimulation

Hydroxyurea (HU) is known to reversibly inhibit DNA synthesis in a variety of cell types. Its primary mode of action is thought to be inhibition of the enzyme responsible for reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates. Eukaryotic cells exposed to HU are stopped in the S phase at the G1/S boundary from continuing through the cell cycle. HU is therefore commonly used to synchronize cell cultures with respect to the cell cycle.

Experiments were carried out to determine: (1) what effects HU might have on the blastogenic response of mouse spleen cells to Con A, (2) whether a relative degree of synchronization could be achieved, and (3) what effects could be observed on incorporation of carbohydrate into glycoproteins and glycolipids.

First, using serial dilutions of HU (Fig. 21), it was found that concentrations below 10^{-4} M actually increased DNA and RNA synthesis measured at 48 hr in Con A stimulated cells. At 10^{-3} M, DNA synthesis became negligible, but RNA synthesis was only reduced by 40% even at 10^{-2}M. The low levels of DNA and RNA synthesis in control cells were not affected at any concentration.

Over the range 10^{-5} - 10^{-3} M, viability at 48 hr was not affected, and the percentage of blast cells decreased only slightly.

Although at 10^{-5} M DNA and RNA synthesis were higher than normal in Con A stimulated cells, incorporation from UDP-[^{14}C]-galactose (in the

Fig. 21. Effects of hydroxyurea on Con A stimulation of mouse spleen cells.

The final concentrations of hydroxyurea, indicated on the abscissa, were added at the beginning of the culture period. Top: Cells were assayed at 48 hr for DNA synthesis (solid lines) and RNA synthesis (dashed lines). Closed symbols, Con A; open symbols, control. 1.0 μCi/ml of thymidine [methyl-^{3}H] was added for the last 4 hr of culture, and 1.0 μCi/ml of [^{3}H]-uridine was added for the last 30 min of culture. Incorporation was assayed as described for DNA synthesis in the legend to Fig. 1. Each value is the mean of duplicate samples from duplicate tubes. Center: Viability was measured by trypan blue dye exclusion, percent blast cells were counted with phase contrast microscopy. Bottom: Incorporation of label from UDP-[^{14}C]-galactose into glycoproteins and glycolipids combined, without 5'-AMP. Cells were washed free of the hydroxyurea immediately before assay. Closed circles, Con A; open circles, control.

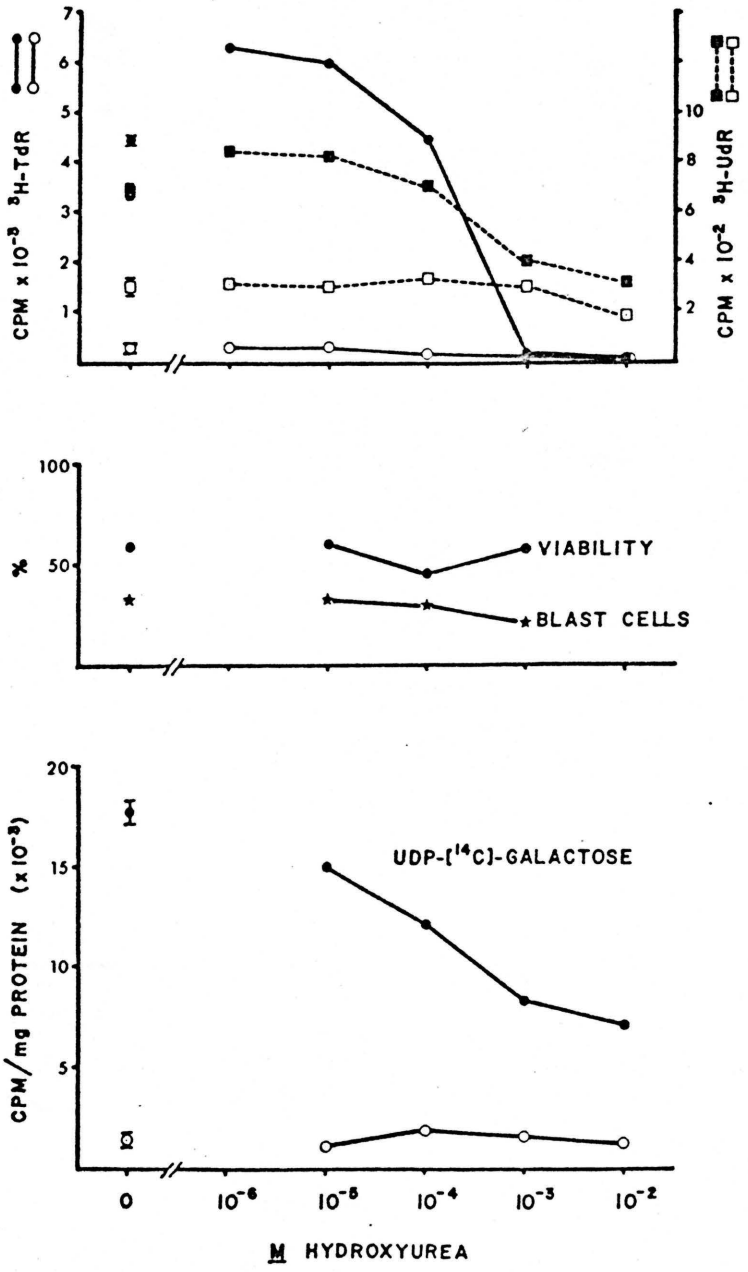


Fig. 21. Effects of hydroxyurea on Con A stimulation of mouse spleen cells.

absence of 5'-AMP) was slightly decreased. At 10^{-2} M it is further decreased to 45% of normal. Incorporation of carbohydrate in control cells was not affected.

For later experiments, a concentration of 10^{-3} M HU was used, as this concentration completely inhibited DNA synthesis, but did not prevent blastogenesis from occurring.

To determine if HU was stopping DNA synthesis at the G1/S boundary reversibly, Con A stimulated cells were incubated with HU and washed free of it at 20 or 44 hr, or not at all (Fig. 22). DNA synthesis was then measured by [^{3}H]-thymidine incorporation between 44 and 48 hr. DNA synthesis was more than three times higher than normal immediately after release from HU at 44 hr, and more than twice as high when released at 20 hr. This would indicate that Con A stimulated spleen cells proceed through the cell cycle, accumulating at the G1/S boundary in the presence of HU and immediately enter the S phase after release.

On the basis of these results, the data in Fig. 23 would seem to indicate that ectomannosyltransferase activity is related to the cell cycle. The bars on the left show the level of incorporation by Con A stimulated cells not treated with HU. When HU was present throughout the culture period (middle bars), and stimulated cells were stopped at the G1/S boundary, incorporation was doubled. If HU was washed out 4 hr before assay, at a time when the maximum number of stimulated cells immediately entered S phase, incorporation was similar to the untreated cells. Thus ectomannosyltransferase activity in Con A stimulated spleen cells is higher in the late G1 phase than in the S phase. This stands in contrast to results obtained by Bosmann (1974) which demonstrated

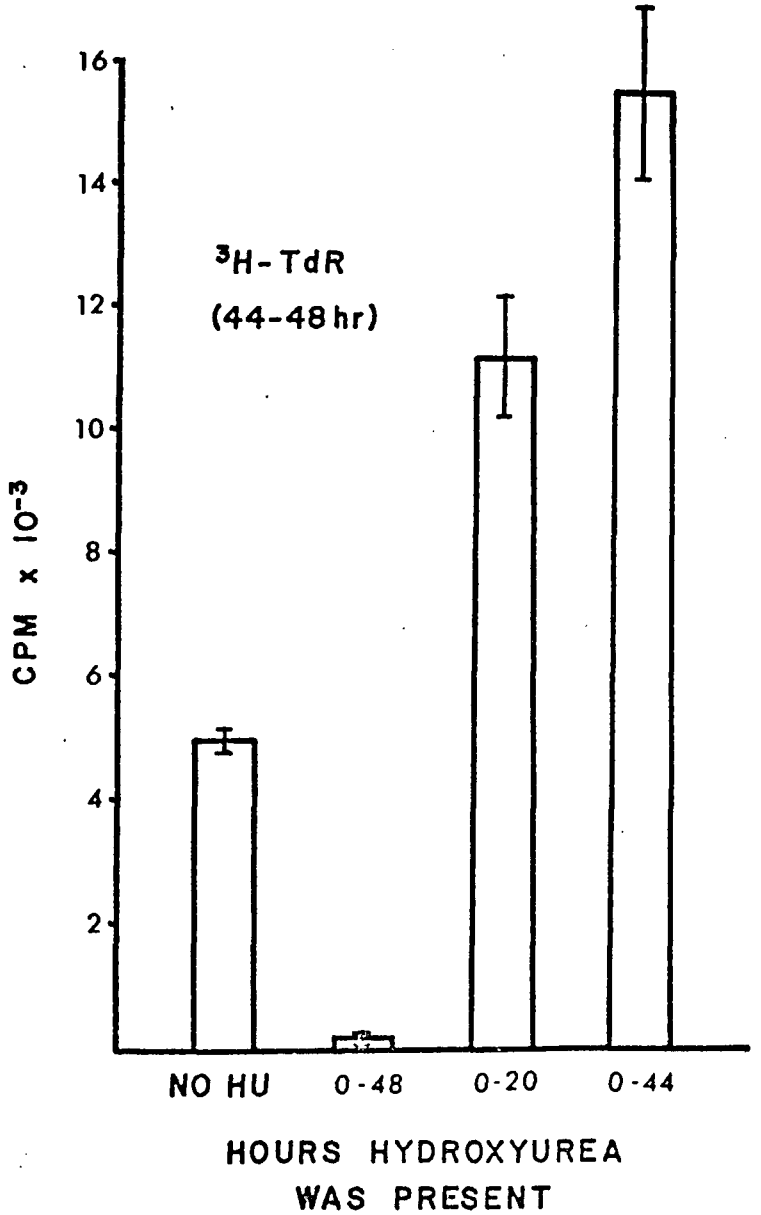


Fig. 22. DNA synthesis at 48 hr in Con A stimulated cells cultured with hydroxyurea.

Incorporation was assayed as described in the legend to Fig. 1. The first bar represents the level of DNA synthesis in Con A stimulated cells without hydroxyurea. The second bar represents the level observed with hydroxyurea present at 0-48 hr. The third represents the level observed when hydroxyurea was washed away at 20 hr. The fourth represents the level observed when hydroxyurea was washed away at 44 hr. Thymidine [methyl-^{3}H] was added at 44-48 hr. In those cases where hydroxyurea was washed away, the medium was replaced with the original culture medium prepared with 10 µg/ml Con A at the beginning of the culture period, and incubated at 37°C with the cell cultures. Each value is the mean $\pm$ the standard deviation of duplicate samples from duplicate tubes.

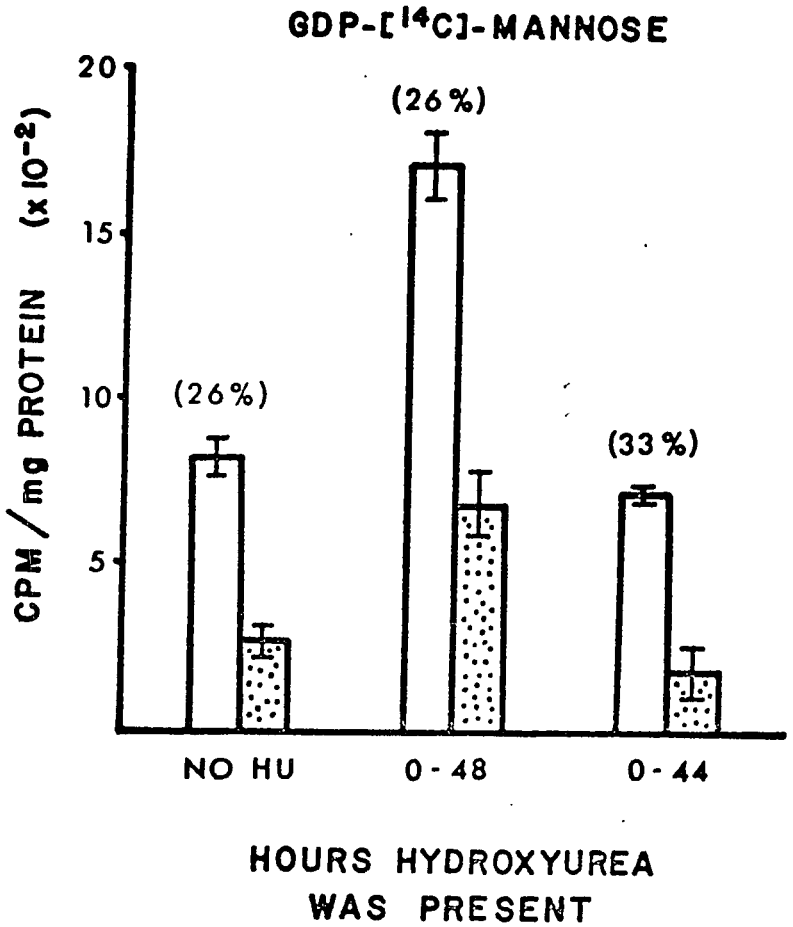


Fig. 23. Incorporation from GDP-[^{14}C]-mannose at 48 hr in Con A stimulated cells cultured with hydroxyurea.

Incorporation was measured into both glycoproteins (open bars) and glycolipids (shaded bars). Bars on the left represent the level of incorporation observed in cells stimulated without hydroxyurea. The center bars represent the level of incorporation observed when hydroxyurea was present at 0-48 hr. Bars on the right represent the level observed when hydroxyurea was washed away at 44 hr. The assay was done at 48 hr. In those cases where hydroxyurea was washed away, the medium was replaced with the original culture medium prepared with 10 µg/ml Con A at the beginning of the culture period, and incubated at 37°C with the cell cultures. Each value is the mean of duplicate samples $\pm$ the range. The numbers in parentheses indicate the percent blast cells in each group of cultures at the time of assay.

maximal activity for several ectoglycosyltransferases during the S phase in a continuously dividing cell line.

Kinetics of Incorporation from GDP-[^{14}C]-mannose

No mammalian glycosylceramides have yet been characterized which contain mannose. However, in a variety of cell types, a mannolipid has been found to be an intermediate in the transfer of mannose from the nucleotide sugar to glycoproteins. This mannolipid has been characterized as a mannosyl phosphoryl polyisoprenol. A second intermediate has also been found which is not soluble in $CHCl_3$:CH_3OH (2:1) or water, but is readily soluble in $CHCl_3$:CH_3OH:H_2O (10:10:3). This is thought to be an oligosaccharide containing N-acetylglucosamine and mannose connected to polyisoprenol by a phosphate or pyrophosphate. The chloroform-methanol soluble mannolipid has been found in lymphocyte homogenates and characterized as mannosyl phosphoryl dolichol (Wedgwood, Strominger, and Warren, 1974).

In order to determine if these intermediates might be synthesized on the surface of Con A stimulated spleen cells, thereby accounting for apparent incorporation of mannose into glycolipids, the following experiment was done. At several time intervals up to one hour, reactions containing GDP-[^{14}C]-mannose and 5'-AMP were extracted with chloroform-methanol. Then each residue was extracted with chloroform-methanol-water. Radioactivity was then measured in both types of extract and the final residue at each time point (Fig. 24).

In contrast to results obtained by others with hen oviduct or mouse fibroblasts, no incorporation could be observed into the residue

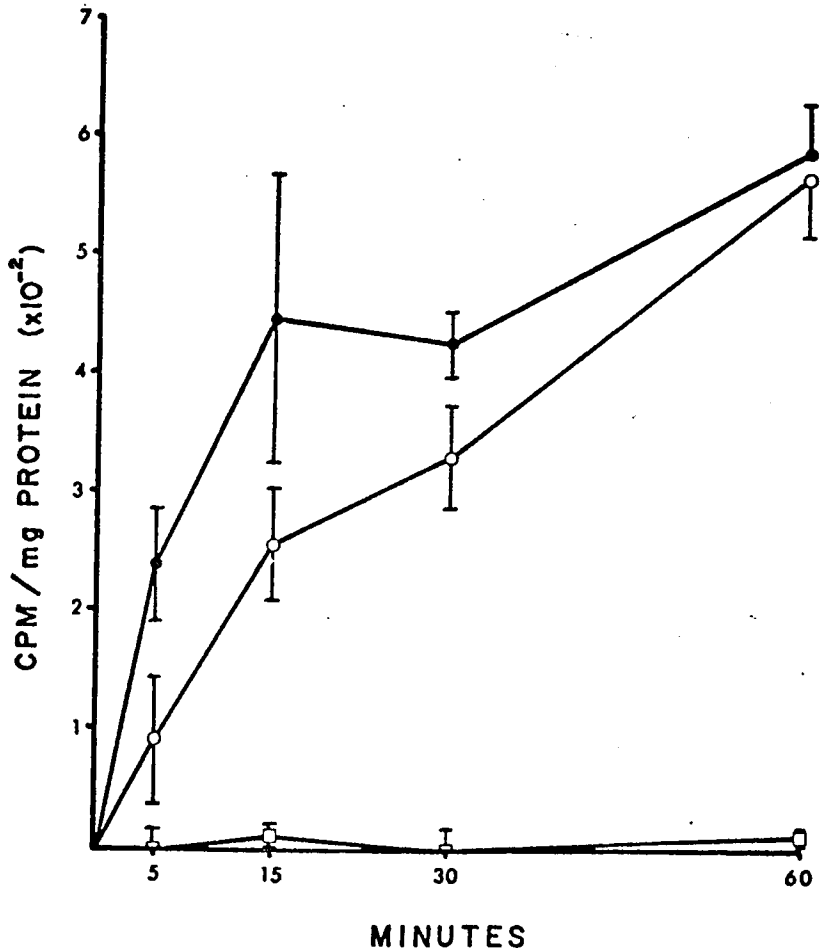


Fig. 24. Incorporation of label from GDP-[^{14}C]-mannose by 48 hr Con A stimulated spleen cells in the presence of 5 mM 5'-AMP.

At the times indicated, reactions were terminated with the addition of 2 ml cold tris buffered saline. The cells were pelleted, the supernate was discarded, and the pellet extracted twice with 2 ml chloroform : methanol (2:1). The extracts were pooled and washed once with 1.5 ml 0.9% NaCl, and once with 2.0 ml of the theoretical upper phase, chloroform : methanol : 0.9% NaCl (3:48:47). The remaining material was then evaporated to dryness in scintillation vials. The cell pellet residues were than extracted twice with 3.0 ml chloroform : methanol : water (10:10:3). These extracts were pooled and evaporated to dryness in scintillation vials. The cell residues still remaining were transferred to glass fiber filter pads and placed in scintillation vials to dry. All fractions were counted in 10 ml scintillation fluor. This is a modification of the procedure described by Waechter, Lucas, and Lennarz (1973). Each value is the mean of duplicate samples ± the range.

material, even though the two lipid intermediates apparently were found. Although the products extracted here were not characterized, the data suggest that only the two lipid intermediates are formed at the cell surface, and not mannosyl glycoproteins.

Ectoglycosyltransferase Activities in Mixed Cell Populations

In attempts to detect alterations of galactosyltransferase activity by cell interaction in the presence of 5'-AMP, several syngeneic and allogeneic cell mixes were tested. L-929 cells (an established cell line derived from C3H fibroblasts), primary C57Bl/6 fibroblasts in the fourth subculture generation (denoted C6F) and C3H macrophages had high levels of activity alone. C6F cells had the highest, C3H macrophages the lowest activity of the three. The freshly harvested spleen cells and thymocytes used had little or no observable activity alone. Various mixtures of these cell types are listed in Table 9. A significant effect (greater than 20% different from the sum of the separate cell types) was observed in only two combinations. This effect was negative in both cases i.e., the activity of the mixtures was less than the sum of each cell type alone. Although the two mixtures in which a decrease was observed are both allogeneic mixtures, six other allogeneic mixtures are presented which show no effect.

The decreased incorporation in the two lymphocyte-fibroblast combinations might be explained by steric hindrance or by actual enzyme-acceptor binding between cells in such a manner as to exclude utilization of extracellular nucleotide sugars for transfer. Still, in the absence

Table 9. Incorporation from UDP-[^{14}C]-galactose by various cell mixtures in the presence of 5'-AMP.[a]

Cell Mixture	Incorporation as a percent of the expected value[b]	Significant Difference[c]
C3H spleen + C57Bl/6 spleen	91	-
C3H thymus + C57Bl/6 thymus	100	-
C3H spleen + C6F fibroblasts	77	+
C3H thymus + C6F fibroblasts	57	+
C57Bl/6 spleen + C6F fibroblasts	104	-
C57Bl/6 thymus + C6F fibroblasts	95	-
C3H thymus + L-929 fibroblasts	84	-
C57Bl/6 thymus + L-929 fibroblasts	94	-
C3H thymus + C3H macrophages	104, 83[d]	-
C57Bl/6 thymus + C3H macrophages	101, 100[d]	-
C3H macrophages + L-929 fibroblasts	121	±
C3H macrophages + C6F fibroblasts	96	-
L-929 fibroblasts + C6F fibroblasts	80	±

a. Spleen and thymus cells were freshly taken from the animal, washed, and used in the assay. Macrophages were cultured four days on collagen and released by collagenase as described in Materials and Methods. C6F fibroblasts were grown originally from minced C57Bl/6 fetuses, 15-17 days old. They were subcultured five times before use in this experiment. L-929 fibroblasts are an established cell line obtained from Flow Laboratories, Inglewood, Ca. They were originally derived from C3H mice. The C6F and L-929 cells had been cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 2 mM glutamine, penicillin and streptomycin. They were released with trypsin (0.025%) + EDTA (10^{-3} M) and then washed and incubated 60 min with fresh medium in suspension before assay. 10^7 spleen and thymus cells and 2.5 x 10^6 macrophages, C6F or L-929 cells were used in 50 µl quantities.

b. The expected value is the sum of the actual cpm incorporated by each cell type alone, not the cpm/mg protein.

c. Arbitrarily chosen as greater than 20% difference from the expected value.

d. Two separate experiments.

of much more data these results cannot be considered to demonstrate an involvement of ectoglycosyltransferases in intercellular interaction.

DISCUSSION

The level of glycosyltransferase activity in a cell is directly related to the cell's synthesis of glycoproteins and glycolipids. Glycosylated proteins and lipids are rare or absent in the cytoplasm, being found exclusively associated with membranes or packaged in secretory vesicles for export. Eylar (1966) hypothesized that glycosylation of a protein predestines that protein for export or integration into the membrane.

Integration of glycoproteins and glycolipids into the plasma membrane is in turn directly related to the expression of cell surface antigens. These antigens are detected by the experimental use of antisera, but are likely to also serve as normal intercellular recognition structures. Many of these have been found to be specific for different stages of ontological development and for different fully differentiated tissues.

Lymphocytes are being shown at the present time to have an incredibly complex array of surface recognition structures, as detected by alloantisera. These are seemingly distributed in a nonrandom way among an equally complex array of subpopulations of lymphocytes -- each presumably serving a unique function in immunological responses.

Some surface structures on lymphocytes are thought to regulate "homing" of lymphocytes in the circulation to various tissues (Gesner and Ginsburg, 1964). Models have been proposed for the involvement of antigenic surface structures in interactions between lymphocytes and (a)

macrophages, (b) other lymphocytes cooperating in an immune response, and (c) chemically modified or virus infected target cells in a cytotoxic response.

In the first model, Shevach and Rosenthal (1973) postulated that macrophages present antigen on their surface to T lymphocytes by physical interaction. The interaction binding site on both cells was thought to be identical to or closely linked genetically to histocompatibility antigen. In addition the lymphocyte expresses an immune response (IR) gene product (the antigen receptor) which is physically related to the interaction binding site.

Second, Katz et al. (1975) found that thymus and bone marrow derived murine lymphocytes can only cooperate in an antibody response if they are syngeneic at the Ia and/or Ib loci within the major histocompatibility region of the genome. Pierce and Klinman (1975) refined this concept to postulate that allogeneic T cells (different at the Ia locus) can interact with an Ia^- subpopulation of B cells in a primary antibody response, but that another population of B cells (possibly derived from the originally Ia^- group) must express a surface Ia antigen identical to the T cell's for a secondary response.

The third model (cytotoxic interaction) states that in order for a T lymphocyte to kill syngeneic cells infected with certain viruses (Doherty and Zinkernagel, 1975) or chemically modified cells (Shearer, Rehn, and Garbarino, 1975) they must recognize an "altered self" surface antigen coded for by the K or D loci of the major histocompatibility region.

Blanden, Hapel, and Jackson (1976) have formulated a speculative theory which postulates a mechanism capable of fitting all the above models. Briefly summarized, this theory states that T cells can only be stimulated by cell surface histocompatibility antigens (effector cells recognize K or D antigens, helper cells recognize Ia antigens) or histocompatibility antigens in conjunction with foreign antigens. Display of the latter type of antigenic pattern is a function of macrophages which are postulated to "process" antigen by glycosylating foreign polypeptides. This would be accomplished by batteries of glycosyltransferases which normally glycosylate histocompatibility antigens. T cell receptors for antigen might then be wholly or partially composed of ectoglycosyltransferases derived from those involved in the production of histocompatibility antigens.

Criteria for Defining Ectoglycosyltransferases -- Application to Lymphocytes

In order to define an ectoglycosyltransferases, the enzyme must be shown to use the added nucleotide sugar directly and transfer it to the acceptor at the cell surface. For these conditions to be met, the nucleotide sugar must not be degraded or transported, and broken cells must be eliminated or accounted for.

Paper chromatography demonstrated that both stimulated and control cells were capable of degrading to the free sugar all substrates tested (Table 1). This contrasts with results presented by Patt et al. (1976), which demonstrated that fibroblasts can degrade nucleotide sugars to sugar phosphate, but not to the free sugar. Degradation was found to be increased in Con A stimulated spleen cell suspensions. This could

have an effect on the increase in activity measured without 5'-AMP (Figs. 2 and 14), although the activity follows similar kinetics when [^{3}H]-galactose is used, suggesting an actual increase in complex polysaccharide synthesis. Increased transport of free sugars may also contribute to the observed increase in activity (Peters and Hausen, 1971; and Averdunk, 1972).

Addition of excess unlabeled free sugar inhibited incorporation from the nucleotide sugars (Figs. 8, 9, 10) indicating that the incorporation occurred after the substrate had been cleaved to the free sugar. Further evidence of an indirect nature was the finding that the radiolabeled carbohydrate of the substrate could be epimerized before incorporation. This was demonstrated by analysis of acid hydrolysates of the reaction products and by the finding that glucosylceramide was labeled by both [^{3}H]-galactose and UDP-[^{14}C]-galactose. Epimerases are soluble cytoplasmic enzymes which use nucleotide sugar as the substrate. Therefore, the sugar was entering the cell prior to transfer. This means either the sugar was cleaved at the cell surface and reactivated intracellularly, or the nucleotide sugar was transported.

Transport of UDP-galactose was found to be negligible compared to uptake of the free sugar (Fig. 11). Patt and Grimes (1974) found that fibroblasts are also unable to transport UDP-galactose. Lack of transport was concluded in both cases on the basis of the observation that the nucleotide sugar did not accumulate in the soluble pool of the cell pellet in the absence of degradation. It could be argued that incorporation occurs at a much faster rate than transport, keeping the intracellular level of nucleotide sugar low. The rate of incorporation in lymphocytes,

however, is not fast compared to fibroblasts, even using the rapidly transported free sugar. It can be calculated from Fig. 11 that 22% of the [^{3}H]-galactose added was transported into the acid soluble pool of the cell pellet, while only 1% of the UDP-[^{14}C]-galactose was transported. Intracellular incorporation is therefore more likely to occur via degradation and uptake of the free sugar than via transport of the nucleotide sugar.

Degradation of the nucleotide sugar substrates could be completely prevented by the addition of 5'-AMP (Figs. 6 and 7). Therefore, incorporation in the presence of 5'-AMP was used as a major criterion for determining the existence of ectoglycosyltransferases.

When investigating ectoglycosyltransferase, broken cells may contribute to apparent surface activity. Patt and Grimes (1974) found that disrupting fibroblasts with the detergent Triton X-100 decreased or did not change incorporation from nucleotide sugars. It was suggested that the detergent might interfere with incorporation, but they concluded that broken cells evidently could not contribute significantly to the incorporation observed in cell suspensions. Triton X-100 also decreased the incorporation observed with lymphocytes (Table 2), but cells intentionally broken by freezing and thawing did show greatly increased incorporation. In the lymphocyte preparations used for this study, no obviously broken cells, or cell debris could be seen microscopically at the time of assay. However, a large fraction of the cells (up to 30 or 40%) had leaky membranes, as evidenced by failure to exclude trypan blue dye. Cells stained by trypan blue are unable to glycolyze, but may retain enzyme activities which do not require free energy. Intracellular incorporation

by these cells seems unlikely for two reasons. First UDP-galactose does not seem to be rapidly accumulated into the intracellular soluble pool, although no account was made of nucleotide sugar which might have been washed back out of leaky cells. Second, and more importantly, when degradation of UDP-galactose was prevented, very little incorporation took place.

Glycosyltransferases Expressed on Different Cells

LaMont, Perrotto et al. (1974) used an exogenous acceptor to demonstrate an ectogalactosyltransferase on neonatal rat thymus, but not spleen cells. This activity increased during blastogenesis of thymocytes induced with Con A. Con A was not blastogeneic for the neonatal spleen cells, and ectogalactosyltransferase activity did not increase in these cells.

By all the criteria discussed above, Con A stimulated mouse spleen cells were shown to express negligible ectogalactosyltransferase activity with endogenous acceptors. An ectoenzyme may be present which can utilize exogenous asialo-agalacto-fetuin (Table 3), but the involvement of broken cells could not be ruled out.

Although incorporation from UDP-[^{14}C]-galactose in the presence of 5'-AMP did not increase in Con A stimulated spleen cells (Fig. 14), it did increase in stimulated lymph node and thymus cells (Fig. 17). This activity was low in thymus cells, however. (See Table 10 for a comparison of spleen, lymph node, and thymus cells.) Lymph node and thymus cells transferred galactose only to glycoprotein acceptors in the presence of 5'-AMP.

Spleen cells stimulated with Con A (T cells), but not LPS (B cells) showed greatly increased incorporation from GDP-[^{14}C]-mannose (see

Table 10. Incorporation from UDP-[^{14}C]-galactose in the presence of 5'-AMP by Con A stimulated and control spleen, lymph node, and thymus cells.[a]

	Con A	CPM/mg protein	
		Glycoprotein	Glycolipid
Spleen Cells	-	594 ± 95[b]	
	+	233 ± 34	288 ± 45
Lymph Node Cells	-	294 ± 15	(-)
	+	1273 ± 182	91 ± 15
Thymus Cells	-	(-)	(-)
	+	478 ± 27	140 ± 22

a. All cells were cultured for 48 hr with 10 µg/ml Con A, and assayed for incorporation from UDP-[^{14}C]-galactose in the presence of 5 mM 5'-AMP. Data for lymph node cells and thymus cells were taken from Fig. 17, and data for spleen cells were taken from a separate experiment. Each value is the mean of duplicate samples ± the range.

b. Glycoproteins and glycolipids not separated.

Table 11 for comparison). Several lines of evidence indicate this occurred at the cell surface: (1) incorporation was not decreased (Fig. 13) when degradation was prevented by 5'-AMP (Fig. 7), (2) incorporation from free mannose was very low compared to the nucleotide sugar (Fig. 13), and (3) mannose was not incorporated into glycoproteins (Fig. 24) suggesting the substrate was not available to the Golgi apparatus. As mentioned in the Results, inhibition of incorporation by excess mannose (Fig. 9) is unlikely to occur via competition for intracellular pools due to the low level of incorporation seen with [^{3}H]-mannose.

Macrophages cultured in vitro exhibited significant incorporation from UDP-[^{14}C]-galactose, UDP-[^{14}C]-glucose, GDP-[^{14}C]-mannose, and UDP-[^{14}C]-N-acetylglucosamine into endogenous acceptors in the presence of 5'-AMP (Fig. 19). Other criteria were not applied to prove that these were ectoglycosyltransferases.

Both macrophages (Fig. 19) and Con A stimulated spleen cells (Fig. 15) incorporated label from CMP-[^{14}C]-N-acetylneuraminic acid after neuraminidase treatment. It isn't known if 5'-AMP is capable of preventing degradation of CMP-N-acetylneuraminic acid.

Relationship of Glycosyltransferase Activity to Biological Parameters

In order to investigate the importance of ectoglycosyltransferases to cellular functions, they must be considered in light of several parameters of cell biology. First, the relationship of ectoglycosyltransferase activity to the cell type (lymphocyte subpopulation) or anatomical location should be noted. Different lymphocyte subpopulations have different functions and some have been found to segregate themselves to an

Table 11. Incorporation from GDP-[^{14}C]-mannose in the presence of 5'-AMP by spleen cells stimulated with Con A or LPS.[a]

	CPM/mg protein
Con A	6883 ± 209
LPS	717 ± 7
Control	660 ± 38

a. Data were taken from Figs. 13 and 16.

extent between different lymphoid organs. Second, the ontological state of the cells should be considered. A variety of surface antigen changes occur in lymphocytes as they develop immunocompetence, and glycosyltransferase activity has been shown to change with differentiation in at least one other system (Weiser, 1973). Third, the mitotic cycle and the physical growth associated with blastogenesis are known to involve membrane alterations and should be related to ectoglycosyltransferase activity. Finally, since the assays in this work were performed largely on cells stimulated by various agglutinins, it should be determined if surface perturbation or agglutination by the lectin are related to the activity measured. Each of these parameters will be considered in turn.

Cell Type

Mouse spleen cells stimulated with Con A (T cells) and by LPS (B cells) both respond with maximal DNA synthesis on day two in culture (Figs. 1 and 16). However, both the level of [^{3}H]-thymidine incorporation and the percent blast cells is somewhat lower in LPS stimulated cultures than in Con A stimulated cultures.

LPS and Con A stimulated cultures differed in this study in their incorporation of mannose into endogenous acceptors in the presence of 5'-AMP. Con A stimulated cells expressed a mannosyltransferase which appears to have a unique mannolipid acceptor (Fig. 24). The lack of an increase in utilization of GDP-mannose in LPS stimulated cells (Fig. 16) may be significant in light of the structural and functional differences between T and B blasts. Although T cell blasts and B cell blasts are morphologically indistinguishable by light microscopy, they have been

shown to differ in their ultrastructure. Shohat et al. (1973) found that LPS stimulated blasts develop much rough endoplasmic reticulum, whereas Con A stimulated blasts contain very little.

Anatomical Location

Con A stimulated T cells from different lymphoid organs in the mouse were also shown to utilize UDP-galactose differently at the cell surface. Stimulated spleen cells transferred very little galactose into either glycoprotein or glycolipid in the presence of 5'-AMP (Fig. 12). Con A stimulated lymph node cells, on the other hand, expressed a galactosyltransferase which used endogenous glycoprotein, but not glycolipid acceptors (Fig. 17). The activity of lymph node cells represented a fourfold increase over control cells at 48 hr, whereas the low activity of spleen cells was unchanged with time. Similar numbers of blast cells occurred in the two types of culture. It is not known if this represents different effects of the lymphoid organs on the same cell, or if distinct subpopulations of T cells were stimulated in the two organs.

Ontological Development

Lymphoid cells in the mouse thymus are 95% immature T cells, which are relatively incompetent in immune responses, and 3-5% mature T cells. Both pools were stimulated by Con A in this study where 32% blast cells were seen after two days. Con A stimulated thymocytes showed incorporation from UDP-[^{14}C]-galactose only into glycoproteins (Fig. 17). The incorporation was low, but still higher than that in unstimulated thymocytes, in contrast to a lack of increase in activity for Con A stimulated spleen cells. Thymocytes were different from both spleen cells and lymph

node cells in that incorporation from UDP-galactose in blasts was not much greater in the absence of 5'-AMP than in its presence. This may indicate a relative lack of cell surface pyrophosphatase activity on thymocytes.

These results do not indicate a specific relationship between the degree of differentiation and ectoglycosyltransferase activity. It is not known to what extent the mature and immature thymocytes contributed to the activity observed. It may be that the same T cells respond in spleen, lymph node and thymus, but interactions with other cells from each organ lead to different expressions of cell surface functions during the blastogenic response.

Blastogenesis, Mitosis, and Agglutination

Experiments comparing the effects of different lectins and LPS on spleen and thymus cells demonstrated that neither blastogenesis, mitosis, nor physical agglutination of lymphocytes is a requirement for stimulation of complex carbohydrate synthesis (Tables 7 and 8).

WGA did not induce an increase in the number of blast cells from either spleen or thymus. AbL and LPS did not induce blastogenesis in thymocytes. All four of these systems did, however, exhibit increased overall complex carbohydrate synthesis with UDP-[^{14}C]-galactose. It was shown that in spleen cells incubated with WGA, much of this synthesis apparently takes place on the cell surface. In the mouse, LPS and Con A are known to stimulate different lymphocyte subpopulations. WGA and AbL may also stimulate unique subpopulations. Distinct subpopulations have been found in spleen and thymus which differ functionally and by

expression of surface antigens. Thus the four combinations above (WGA with spleen and thymus, AbL and LPS with thymus) are likely to be affecting different cells, but it seems safe to conclude that glycosyltransferase activity can be stimulated in the absence of blastogenesis.

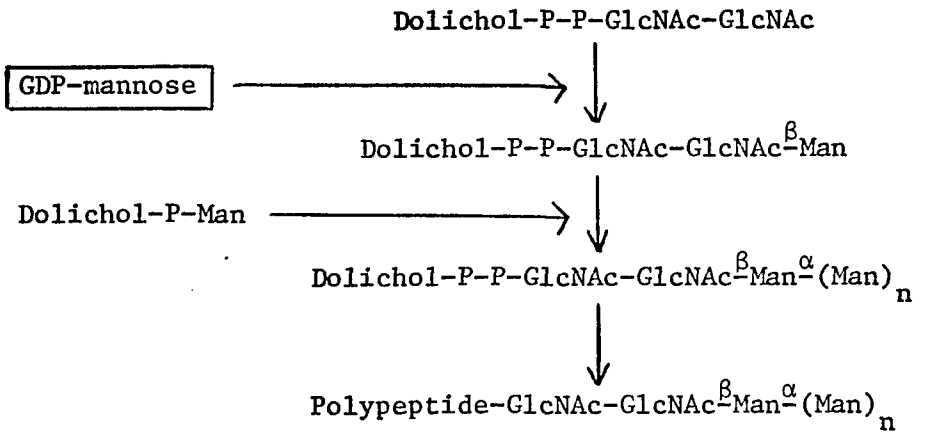
Likewise, WGA, AbL and LPS did not initiate DNA synthesis in thymocyte cultures, but did increase incorporation of galactose. Thus glycosyltransferase activity can be stimulated in the absence of mitogenesis.

DNA synthesis was further shown to be unnecessary for stimulation of glycoprotein and glycolipid synthesis in the experiments using hydroxyurea with Con A stimulated spleen cells. In the complete absence of DNA synthesis, intracellular incorporation from UDP-galactose still took place (Fig. 21). Partial synchronization with hydroxyurea indicates that cell surface mannosyltransferase activity is preferentially expressed in the late G1 phase as compared to the S phase (Fig. 23).

LPS did not agglutinate either spleen cells or thymocytes, but did increase intracellular galactose incorporation in both (Fig. 16 and Table 8). Therefore agglutination is not necessary to stimulate complex carbohydrate synthesis.

Utilization of GDP-mannose by Con A Stimulated Spleen Cells

Incorporation of mannose into glycoproteins is thought to proceed via the sequence:



(Taken from Lennarz, 1975)

Dolichol phosphomannose has been found in many cell types, including human lymphocytes (Wedgwood et al., 1974), and is soluble in chloroform-methanol (2:1). Another polyisoprenol linked oligosaccharide containing mannose has been found in hen oviduct which is insoluble in chloroform-methanol but soluble in chloroform-methanol-water (10:10:3).

Although not characterized in this study, two classes of product labeled by GDP-[^{14}C]-mannose in the presence of 5'-AMP were found in Con A stimulated spleen cells (Fig. 24). One was soluble in chloroform-methanol and the other in chloroform-methanol-water, similar to the two polyisoprenol derivatives mentioned above. These products in Con A stimulated spleen cells appear to be made using GDP-mannose at the cell surface and do not seem to transfer mannose onto glycoproteins in lymphocyte suspensions.

Since increased cell surface incorporation from GDP-mannose was not found in LPS stimulated spleen cells or Con A stimulated thymocytes, formation of the mannolipid intermediates may serve a unique function in Con A stimulated spleen cells.

Possibility of a Role for Ectoglycosyltransferases in Cellular Interactions

Demonstrating the presence of ectoglycosyltransferases on cells is much easier, in all its complexity, than demonstrating their direct involvement in cellular interactions. Some suggestive evidence has accumulated to say that they can play a functional role at the cellular level (reviewed by Shur and Roth, 1975). However, most of the evidence is of a correlative nature, relating enzyme activity to a particular state of the cell (e.g., phase in the mitotic cycle, cell density, transformation, differentiation, etc.). This type of evidence cannot be interpreted in terms of cause and effect. It is also limited in its viewpoint (as most scientific investigation is forced to be) in that only one of many possible factors related to the phenomenology is studied at a time.

In this investigation, no attempt has been made to demonstrate a particular function for ectoglycosyltransferases on lymphocyte surfaces. Rather, more correlative evidence with a limited viewpoint has been gathered which can also be interpreted as suggesting, but not proving, a role for these ectoenzymes.

The evidence can be summarized as follows: (a) Ectoglycosyltransferase activity was expressed differently on different cells which have different functions. In general, surface activity was low in lymphocytes compared to fibroblasts or marcophages. This may be relevant to the fact that lymphocytes are a free floating cell in vivo. (b) Increased glycosyltransferase activity was not strictly correlated with the growth which occurs during blastogenesis. Thus it seems not to be a

nonspecific anabolic process related only to physical growth. (c) Although galactosyltransferase activity was not strictly correlated with the commitment to DNA synthesis, mannosyltransferase activity was related to the cell cycle. (d) The presence of an ectogalactosyltransferase on Con A stimulated spleen cells which is only detected with an exogenous acceptor and cannot use intracellular UDP-galactose suggests that its role on the surface cannot be repair or synthesis of surface molecules.

Because the plasma membrane is constructed by the Golgi apparatus, the possibility has been suggested (LaMont, Perrotto et al., 1974) that glycosyltransferases may be nonspecifically carried to the cell surface from that organelle, serving no further function on the cell surface. Changes detected in surface activity may only reflect changes in Golgi activity. During lymphocyte stimulation, a barely detectable Golgi is transformed into a highly active complex. New membrane is rapidly produced as the cell size increases. Results presented here, however, suggest that the expression of ectoglycosyltransferases during blastogenesis seems to be restricted, or selective.

If ectoglycosyltransferases are not accidental, and are different on different cells, and in different states of the same cell, one other alternative must still be considered for their function. It could be that they are expressed selectively in order to produce other surface molecules which themselves mediate cellular interactions.

Many specialized functions of plasma membranes, beyond simple control of nutrient uptake and waste excretion, have been identified recently. We are still a long way from understanding the mechanisms behind them.

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