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Altered patterns of glycosylation on rat lymphocytes associated with activation

P. SUTTON, R. W. STODDART* & I. V. HUTCHINSON *Immunology Research Group, Department of Cell and Structural Biology and *Department of Pathological Sciences, University of Manchester, Manchester*

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SUMMARY

The expression of cell-surface carbohydrates on rat lymphocytes was investigated by flow cytometry using a panel of lectins. A small group of lectins was identified, all with a main binding requirement of *N*-acetylgalactosamine that bound to all B lymphocytes but only to activated T lymphocytes expressing the interleukin-2 (IL-2) receptor (as shown by staining with the monoclonal antibody OX39). Studies demonstrated that five of these lectins competed for the same binding site, while others did not. With the knowledge of the binding requirements of these lectins, a structure can be deduced for the carbohydrate moiety which appears on T lymphocytes when activated.

INTRODUCTION

Glycoprotein receptors and ligands on the surface of lymphocytes play an important role in immune regulation. With the development of monoclonal antibodies it has been possible to separate and purify many of these molecules, allowing for detailed peptide sequencing and, in the case of the histocompatibility antigens, to determine the three-dimensional conformation of the molecule, based on its protein composition, crystallography and electron density mapping.[1] The carbohydrate component of cell-surface glycoproteins, however, has been relatively neglected, even though there is clear evidence that this can play a significant functional role.[2-5]

Activation of a cell leads to alteration of the expression of cell surface receptors and ligands. In the resting state, surface molecules are positioned to facilitate basal levels of nutrient uptake and the shedding of cellular products, and to monitor the environment for effector molecules such as hormones and growth factors which may trigger the cell to alter its function and become 'activated'. In the case of T lymphocytes, activation signals include interaction with antigen-presenting cells involving cell–cell adhesion molecules, the presentation of antigen in association with major histocompatibility complex (MHC) molecules to T-cell receptors and the binding of cytokines to specific cell-surface receptors.

As the majority of the molecules involved in these reactions are glycoproteins, it is likely that altered glycosylation of T-cell surfaces will accompany cellular activation and might provide a means by which immune function could be manipulated. Since the outermost parts of cellular surfaces, including those of lymphocytes, are particularly rich in carbohydrate[6] and saccharides generally occupy much larger hydrated volumes than peptides of equivalent molecular weight,[7] it is likely that alteration of surface glycans will have extensive consequences for immune and other cellular functions.

Carbohydrate-binding proteins or glycoproteins (lectins) have already been shown to be capable of distinguishing lymphocyte subpopulations. The soybean lectin binds to the B-cell form of leucocyte common antigen,[8] while the lectin from *Vicia villosa*, once thought to bind specifically to cytotoxic T cells,[9] may be selective for contra-suppressor cells.[10]

Here we have used a selected group of lectins to demonstrate that T lymphocytes exhibit altered glycosylation when activated in an allogeneic mixed lymphocyte reaction.

Abbreviations: DAB, Dulbecco's A + B salt solution; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; FCS, foetal calf serum; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; HBSS, Hanks' balanced saline solution; lectins, all lectin abbreviations are listed in Table 1; mAb, monoclonal antibody; MLR, mixed lymphocyte reaction; Ser, serine; Thr, threonine.

Correspondence: Professor I. V. Hutchinson, Immunology Research Group, Department of Cell and Structural Biology, School of Biological Sciences, University of Manchester Medical School, Oxford Road, Manchester M13 9PT, U.K.

MATERIALS AND METHODS

Lectins

All lectins were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) as biotin or fluorescein isothiocyanate (FITC) conjugates. Lectins were dissolved at the concentrations stated in 0·05 M Tris-buffered saline plus 1 mM calcium chloride, and stored at 4°.

The lectins used, with their standard abbreviations, are listed in Table 1.

Table 1. Lectins and their dominant saccharide-binding specificities

Approximate sugar specificity	Source of lectin/agglutinin	Abbreviation
N-acetylgalactosamine	*Bauhinia purpurea*	BPA
	Griffonia simplicifolia	BSI-A4
	Caragana arborescens	CAA
	Dolichos biflorus	DBA
	Helix aspersa	HAA
	H. pomatia	HPA
	Glycine max	SBA
	Wisteria floribunda	WFA
Mannose	*Pisum sativum*	PSA

Cell preparation

The superficial cervical and mesenteric lymph nodes of AO ($RT1^u$), or AO × PVG ($RT1^u \times RT1^c$) F_1 hybrid rats, were teased gently to release cells into suspension. These rats were bred in the Animal Unit of the University of Manchester Medical School. The lymphocytes were washed twice with Hanks' balanced saline solution (HBSS) and resuspended at 10^7 cells/ml in HBSS for fluorescence-activated cell sorter (FACS) staining.

Cell activation

Lymphocytes were normally activated using the mixed lymphocyte reaction (MLR). (AO × PVG) F_1 stimulator cells were mixed with an equal number of AO responder cells to a final concentration of 10^7 cells/ml in RPMI-1640 culture medium with 7% (v/v) foetal calf serum (FCS), streptomycin (45 μg/ml), penicillin (45 μg/ml), kanamycin (90 μg/ml) and 10^{-8} M 2-mercaptoethanol. Cultures were incubated at 37° in a humidified CO_2 incubator. Occassionally, activated T cells were generated by culturing AO cells with concanavalin A (Con A) at 5 μg/ml in the above medium and conditions for 3 days. Before staining, dead cells were removed by spinning at 600 *g* for 20 min over histopaque 1083 (Sigma Chemical Co.).

FACS analysis

All staining for flow cytometry was performed at 4°. 10^6 cells (100 μl aliquots) were incubated for 30 min with 50 μl of lectin, used at a predetermined optimal concentration, with or without 50 μl of mouse ascites anti-rat leucocyte monoclonal antibodies (Seratec, Oxford, U.K.). The monoclonal antibodies (mAb) used were OX19 (T-cell marker), OX8 (CD8 marker), W3/25 (CD4 marker), OX6 (class II MHC marker—which in a rat lymph node suspension binds predominantly to B cells) and OX39 [interleukin-2 (IL-2) receptor activation marker]. The cells were washed twice in Dulbecco's A + B salt solution (DAB) with 2% (v/v) foetal calf serum (FCS) and 0·02% (w/v) sodium azide (DAB-2-azide) and incubated for a further 30 min with 20 μl of phycoerythrin-streptavidin (1/50 dilution of a stock solution from Sera Lab, Crawley Down, Sussex, U.K.) plus 50 μl of a 5% (v/v) solution of FITC conjugated rabbit anti-mouse IgG (from Dako, High Wycombe, Bucks, U.K.) in 80% (v/v) DAB and 15% (v/v) normal rat serum. After washing once with DAB-2-azide and once with DAB, the cells were fixed in 1% (w/v) paraformaldehyde and stored in the dark at 4° to await analysis on a Becton Dickinson fluorescence activated cell sorter (FACS) FACScan machine (Becton Dickinson, Mountain View, CA). Most samples were analysed within 24 hr and no changes in specimens were observed over much longer storage periods. FACS analysis was performed on a population of cells, electronically gated to exclude dead cells and debris.

These experiments have been performed many times, with some lectins used as positive (PSA) and negative (DBA) controls for other lectins in the panel. Binding patterns were generally very consistent from one experiment to the next. The data shown in the figures give a composite and representative view of the binding of lectins.

Cell depletion

In order to deplete B lymphocytes prior to FACS staining, cell suspensions were incubated at 4° for 30 min with 100 μl each of OX6 and OX12 (mouse anti-rat kappa chain) ascites antibody at a 1/10 dilution for each 10^8 cells. Monoclonal antibody (mAb)-treated cell suspensions were then rotated at 4° for 30 min with an equal volume of magnetic goat anti-mouse IgG particles (Metachem Diagnostics, Northampton, U.K.) that had previously been washed three times with RPMI-1640 medium with 20 mM HEPES buffer (Gibco, Paisley, Renfrewshire, U.K.) and 5% (v/v) FCS. Washing involved resuspending the particles in the appropriate medium and pulling them to the side of the containing tube with a magnet. The medium could then be removed with a pasteur pipette. Cells unbound by magnetic particles after incubation were obtained by washing the cell/particle suspensions with HBSS, as described above and keeping the wash solutions. The carry-over of cells bound to magnetic particles was reduced by treating the wash solutions with the magnet again.

RESULTS

The concentration of lectin used for staining proved to be critical for discrimination of high- and low-staining populations (data not shown). The figures presented show patterns of lectin binding to resting and activated rat lymphocytes at the optimal lectin concentrations.

Lectin binding to unactivated lymphocytes

Figure 1 shows representative binding profiles of the lectins for resting rat lymphocytes. DBA did not bind to unactivated lymphocytes above background levels, at concentrations of up to 500 μg/ml (data not shown). The other lectins with specificity for GalNAc had binding patterns which were very similar to each other. WFA, HPA, SBA, HAA and BSI-A4 gave a large negative population and a positive peak consisting of 30–35% of gated cells (Fig. 1a). BPA and CAA stained two positive populations identical in proportion to those in Fig. 1a, but with higher fluorescence. Dual-staining profiles with these lectins and mAb demonstrated that the negative or very low-staining subpopulation of resting lymphocytes consisted of $OX19^+$ T cells (Fig. 1b), including both $CD4^+$ and $CD8^+$ lymphocytes (Fig. 1c and d), while the positively staining subpopulation consisted of $OX6^+$ B cells (Fig. 1e). The OX39 (IL-2R) mAb was used to confirm that the T cells were unactivated (Fig. 1f).

On some occasions, a small percentage of T cells prepared from fresh lymph nodes were positively stained with these lectins. It was noted that the lymph nodes of the rats used in these particular experiments were enlarged, which suggested

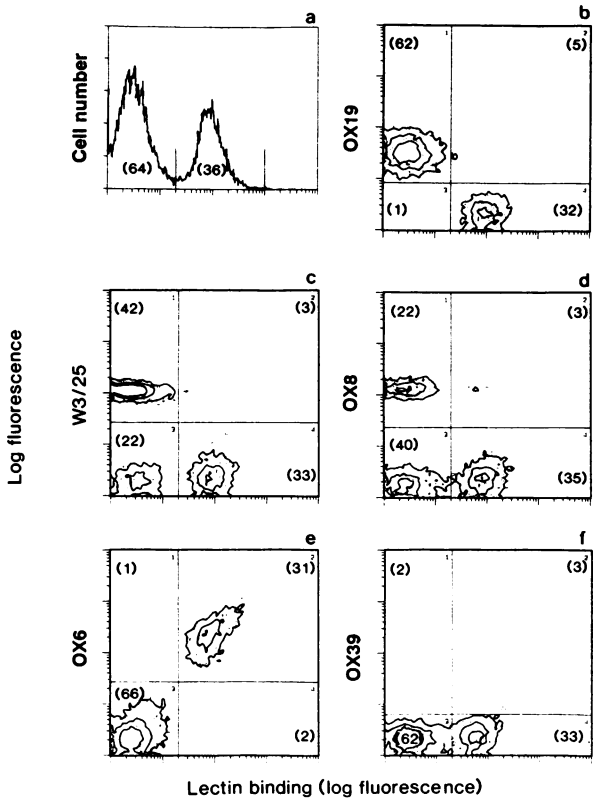

Figure 1. Attachment of lectins and monoclonal antibodies to unactivated rat lymphocytes. The profiles shown were obtained using the lectin WFA (10 μg/ml), but they are also representative of SBA (25 μg/ml), BPA (50 μg/ml), CAA (10 μg/ml), BSI-A4 (1 μg/ml), HPA (50 μg/ml) and HAA (10 μg/ml). The absolute level of fluorescence produced by these lectins varied, but their relative proportions with monoclonal antibody were constant. The numbers in parentheses indicate the percentage of cells located in each quadrant.

that the observed changes in lectin binding could reflect inherent activation. To investigate this hypothesis, the dual staining with lectins and mAb was repeated on lymphocytes activated for 5 days in the allogeneic MLR.

Lectin binding to activated lymphocytes

MLR-activated lymphocytes exhibited an increased proportion of cells binding the lectins WFA, SBA, BPA, CAA and BSI-A4 (Fig. 2). Since these lectins bind to virtually all B cells in the resting state (Fig. 1e), any increase in the number of cells stained must have arisen from changes in the T-cell population. Upon activation in the MLR, a considerable number of $OX8^+$ and $W3/25^+$ cells could be stained by most of the lectins tested (Fig. 2). The majority of lectins tested which had bound to unactivated B lymphocytes also bound to T cells that stained with OX39 (IL-2R) mAb (Fig. 2f), indicating that the increase in ligand expression occurred upon activated T cells, of both $CD8^+$ (Fig. 2c) and $CD4^+$ (Fig. 2d) phenotype.

The exceptions to the general pattern of increased lectin binding to T cells were HPA and HAA which did not identify a positively stained population when activated cells were tested. However, these lectins agglutinated the cells and it was not possible to analyse this population using the flow cytometer.

Acquisition of lectin binding to T lymphocytes

The change of lectin binding with time in mixed lymphocyte culture was studied. Cells were sampled daily from a MLR, depleted of B cells and double stained with mAb and the lectin WFA. This lectin was chosen as it consistently gave clear, distinct binding patterns. The expression of the ligand for WFA on T cells increased during the MLR, becoming maximal at about Days 4/5 and coincided with the expression of the IL-2 receptor (Fig. 3).

Lectin competition for WFA binding sites

The GalNAc-specific lectins WFA, SBA, BSI-A4, BPA and CAA have been shown to behave in a very similar manner, binding to B cells and exhibiting new or increased binding to activated T cells. Thus, the possibility arose that these lectins had detected a single structure on T cells that only appeared upon activation, although they may have bound to different saccharide sequences upon the same molecule.

In an attempt to address this hypothesis, resting unfractinated and activated T cells (Day 3 Con A lymphoblasts, depleted of B cells before staining) were pretreated with unconjugated lectins for 30 min, on ice, before being stained

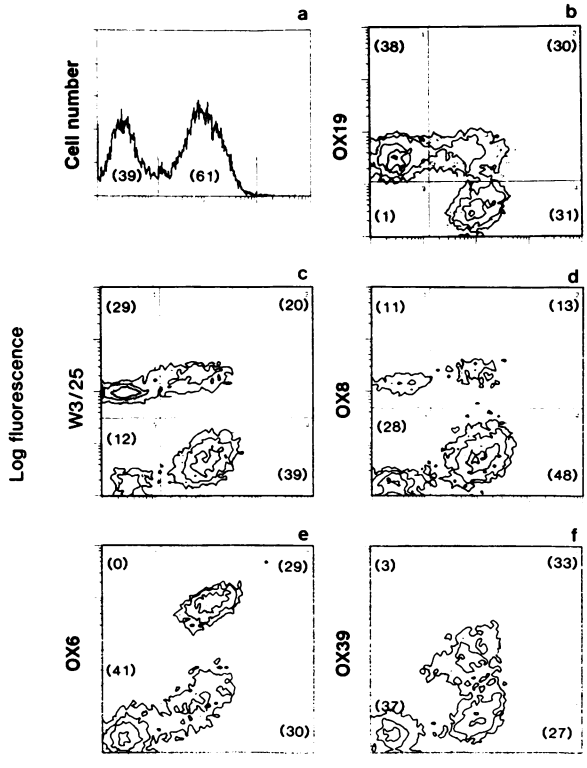

Figure 2. Attachment of lectins and monoclonal antibodies to Day 5 MLR activated rat lymphocytes. The profiles shown were obtained using the lectin WFA (10 μg/ml), but they are also representative of SBA (25 μg/ml), BPA (50 μg/ml), CAA (10 μg/ml) and BSI-A4 (1 μg/ml). The absolute level of fluorescence produced by these lectins varied, but their relative proportions with monoclonal antibody were constant. The numbers in parentheses indicate the percentage of cells located in each quadrant.

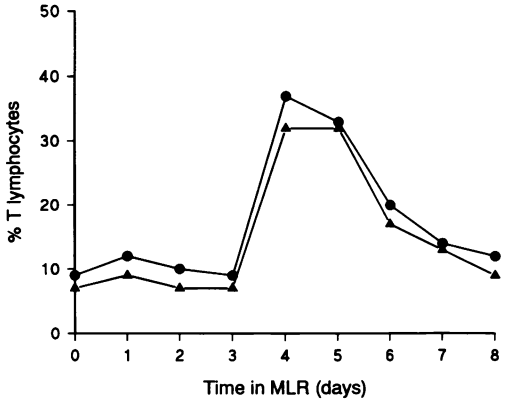

Figure 3. Changes in attachment of the lectin WFA to T-cell subsets upon activation in the MLR. MLR cultures were sampled daily, depleted of B lymphocytes and stained with WFA lectin and/or OX39 mAb: % of T cells binding WFA (●); % of T-cells binding OX39 (▲).

with biotinylated-WFA as above. Con A lymphoblasts were used instead of MLR-activated cells to ensure that a high proportion of T cells were in the activated state. Previous experiments had shown that the increase in lectin binding occurred with T cells activated by either method (data not shown). Competition for the binding site of WFA by the unconjugated lectins was assessed by the reduction of fluorescence measured on FACS analysis. Controls were the lectins DBA, which did not bind to rat lymphocytes, and PSA which bound strongly to all lymphocytes but in a pattern different from those of the lectins listed above.

The results shown in Fig. 4 demonstrated that CAA, BSI-A4, SBA, and BPA competitively blocked the glycan(s) to which WFA bound on both B cells and activated T cells, whereas others including PSA, DBA, HAA and HPA did not.

DISCUSSION

Previous workers have used lectins to subdivide lymphocytes, including murine thymocytes with DBA[11] and human peripheral blood lymphocytes with LCA and WGA,[12] while Schoenbeck *et al.* claimed that VVA separated murine Peyer's patch T cells into IL-5- and IL-2-producing subsets.[13] Here, flow cytometric analysis has allowed both the numbers of leucocytes binding lectins and the amounts of lectin bound to be measured.

An extensive survey of lectins of various specificity using flow cytometric analysis revealed that some lectins bind to subpopulations of lymphocytes. DBA did not bind to unactivated lymphocytes, CAA and BPA gave two peaks, both of which moved to higher fluorescence on titration, while WFA, HAA, HPA, SBA and BSI-A4 gave a single fluorescent peak representing 30–35% of the total cell population and a non-fluorescent peak containing the remainder (Fig. 1). The higher-staining population always contained all B cells and sometimes some T cells. Between experiments, an association was noted between enlargement of the cervical lymph nodes and lectin binding to the T lymphocytes obtained from them. This suggested that immune activation *in vivo* could lead to changes in the expression of cell-surface carbohydrates.

In support of this, when deliberately activated lymphocytes were stained with these same lectins, DBA was unchanged, WFA, SBA, CAA, BPA and BSI-A4 gave an increased positive

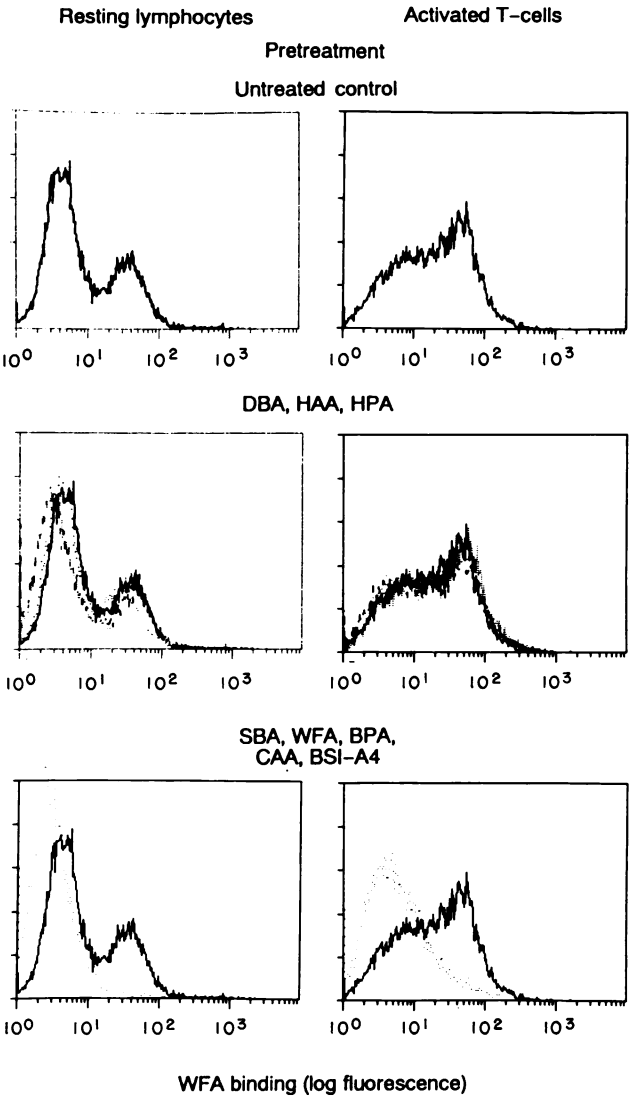

Figure 4. Lectin competition for the WFA binding site on resting and activated lymphocytes. Cells were pretreated with the lectins listed, unconjugated at 100 μg/ml (BSI-A4 was used at 10 μg/ml to prevent cell agglutination which occurred at higher concentrations) prior to staining with biotinylated WFA at 10 μg/ml. The profiles shown are representative of all the lectins listed within a group which behaved the same.

or high-staining peak (Fig. 2), while HAA and HPA staining was reduced. Those lectins which stained MLR-activated T cells were the same as those which bound to the T cells from rats with enlarged lymph nodes. The exceptional lectins, HPA and HAA, agglutinated cells, as shown by the forward/side scatter profile, leading to a selective exclusion of these cells by the electronic

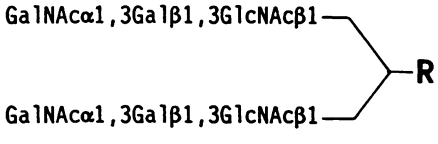

Figure 5. Proposed structure to account for the binding of the lectins WFA, SBA, BPA and BSI-44.

gate used in the analysis (data not shown). This would account for the lack of a positive peak when these lectins were used to stain activated lymphocytes. Their peculiarity seems, therefore, to be artefactual with their underlying behaviour being the same as with the other lectins. However, the study of competition between lectins for binding sites indicated that these two lectins may bind to a structure(s) distinct from that bound by the others. It is possible that they could bind to the same structure if either (1) they also bind to a second glycan (not interactive with WFA) with a higher affinity than for the WFA site or (2) if WFA has a much higher affinity than HPA and HAA and so displaces them.

To summarize, the '*N*-acetylgalactosamine-binding' lectins WFA, SBA, BSI-A4, BPA and CAA have been shown to behave in a very similar manner to each other in their binding to lymphocytes, both in the resting state and after activation. The OX39 (IL-2R) lectin double staining and the time course studies using WFA (Figs 1–3) proved that the expression of a ligand or ligands on T cells for these lectins was restricted to activated cells. It is possible that the lectins WFA, SBA, BPA, CAA and BSI-A4 are interacting with a common structure on B cells and on activated T cells, although it is not known whether the molecule that bears this structure is the same on the two cell types. The observations that HAA and HPA have similar binding patterns to WFA, but do not interfere with its binding, could be explained if they bind to a different segment of the molecule and do not block the WFA receptor site.

Presence of *N*-acetylgalactosamine

The lectins used in this study have known dominant binding requirements for *N*-acetylgalactosamine, as largely determined by binding inhibition analysis with sugars and small glycans. In practice, the structural requirements for high-affinity binding are more exacting than this and in most cases involve several adjacent sugars, so that discrimination between oligosaccharides with different lectins is more extensive than this crude grouping would imply. DBA binds with high affinity to glycans containing the sequence GalNAcα1,3(Fucα1,2)Galβ1,4GlcNAcβ1- but shows very low affinity towards related glycans that lack the fucosyl residue.[14–16] CAA has a requirement for α-*N*-acetylgalactosamine,[17] but the further constraints on its binding are poorly defined. BPA binds with high affinity to the glycan Galβ1,3GalNAcα1- and with lower affinity to GalNAcα1,3Galβ1,3GlcNAcβ1-.[18,19] The behaviour of these two lectins suggests they may have two ligands on the lymphocytes, one universal to all cells and one similarly distributed to those for the other lectins discussed here. HPA has a binding requirement for *N*-acetylgalactosamine alone, with a preference for the α-anomer[16] and HAA is similar. SBA shows a higher affinity interaction with GalNAcα1,3Galβ1,3GlcNAc- than with GalNAcα1-Ser/Thr and a weaker interaction with β-*N*-acetylgalactosamine. WFA, distinctively, has a very high affinity for GalNAcα1,6Galβ1- but its affinity constant for glycans with *two* non-reducing termini of GalNAcα1,3Galβ1- is higher than for those with a *single* GalNAcα1,6Galβ1- terminus.[20] BSI-A4 is highly selective for terminal α-N-acetylgalactosamine.[21]

A knowledge of the binding specificities of these lectins allows deduction of a possible partial structure for the glycan which appears on activated T cells.

Proposed structure of lymphocyte glycans

Where the cell populations show similar behaviour with two lectins of differing chemical specificity, several possible explanations arise: (1) both lectins may be binding to separate, or overlapping, sites on a single glycan, (2) they may be interacting with two separate glycans attached to a single polypeptide, or (3) they may be attaching to two separate glycans on separate polypeptide chains which are expressed in parallel. The latter might arise by more than one mechanism.

The simplest glycan moiety consistent with the known binding requirements of WFA, SBA, CAA, BSI-A4 and BPA is a branched structure with two arms of the form GalNAcαl, 3Galβ1,3GlcNAcβ1- (Fig. 5). Hence a single type of glycan sequence could account for the binding of WFA, as well as BPA, CAA, SBA, and BSI-A4. In Fig. 5 the galactose is shown in β1,3-linkage (type 1) to *N*-acetylglucosamine, to correspond most closely to the binding requirements of the lectins involved, although the possibility that it is in the β1,4-linked (type 2) form cannot be excluded. It is possible that such variations in linkage could occur without necessarily effecting the binding of most of these lectins, with the possible exception of BPA.

After differentiation a cell has functions different from those of its precursor. This generally implies a change in the expression and disposition of molecules at the cell surface. Changes in T-cell activation antigens are examples of this. Fleischer found an antigen of 103,000 MW expressed on all types of proliferating human T cells.[22] LeFrancois and co-workers have identified carbohydrate differentiation antigens that are specific for activated murine cytotoxic T cells, their expression being regulated by IL-2[23] and related to cytolytic activity.[24] Most interestingly, by using a panel of lectins on cell lines, they found that T-cytotoxic cells had an increased expression of GalNAc-containing saccharides on activation, which did not occur on T-helper cells.[23,24] Even though these observations are at slight variance with those reported here (which may be explained by species variation or by the use of cell lines), it is interesting to note that T-cell activation and function was once again associated with changes in the expression of GalNAc-containing glycans. It may be possible to relate the generation of this new glycan moiety with changes in cellular function.

Brown and Williams demonstrated that SBA binds to the B-cell form of CD45R leucocyte common antigen.[8] As this lectin, amongst others, competitively inhibits the binding of WFA to β lymphocytes, it is highly likely that this is the ligand for all these lectins on the B cell. This form of the CD45R molecule is not expressed on T cells, but splice variants of it are, so it is possible that the altered glycosylation observed on T-cell activation may occur on the leucocyte common antigen. However, given the precise correspondence between the appearance of the glycan to which WFA and the other lectins bind and the expression of the IL-2 receptor (Fig. 3), this molecule must also be considered as a potential receptor for these lectins. If this proves to be the case, it would provide a method for detecting the IL-2 receptor and T-cell activation, without the use of monoclonal antibodies.

We have demonstrated that T-cell activation leads to the expression of at least one glycan that is absent or expressed at low levels on resting T lymphocytes. The function of this glycan is, as is the case for most glycans, unknown. It may serve directly as a recognition or adhesion molecule; it may contribute to

maintaining the shape of the molecule to which it is attached, or to protect it from enzymic degradation. Work is currently underway to study, in detail, the changes in binding of lectins with different binding specificities to T cells upon activation and to identify biochemically the molecule to which the lectins described here are binding.

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