Flow cytometric analysis of lectin binding to human peripheral blood lymphocytes

George BLACKLEDGE,* Brent VOSE,† Andrew J. MORRIS,* Derek CROWTHER* and John T. GALLAGHER*

*Cancer Research Campaign, Department of Medical Oncology, Christie Hospital and Holt Radium Institute and Manchester University, Wilmslow Road, Manchester M20 9BX, U.K., and †Department of Immunology, Paterson Laboratories, Christie Hospital, Manchester M20 9BX, U.K

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The binding of fluorescein-conjugated lentil lectin and concanavalin A to the surface membrane of human peripheral blood lymphocytes was studied by flow cytometry. The lymphocytes bound 3-fold more lentil lectin molecules compared with concanavalin A molecules and lentil lectin binding approached saturation at a much lower concentration than did that of concanavalin A. Lentil lectin identified two groups of lymphocytes: a low-binding T-cell fraction and a high-binding B-cell-enriched fraction. Concanavalin A did not discriminate between these populations in unseparated lymphocytes. Competition studies indicated that lentil lectin and concanavalin A were bound to different sites on the lymphocyte surface, although about 50% of lentil lectin sites were in close proximity to concanavalin A sites.

The carbohydrate residues of glycoproteins and glycolipids are located on the external face of the plasma membrane where they may mediate interactions with neighbouring cells and the extracellular matrix. The asymmetric distribution of membrane carbohydrates could be of significance in cell–cell recognition since intercellular adhesion of lymphocytes may be regulated by major histocompatibility-complex-determined protein–carbohydrate interaction at cell surfaces (Parish *et al.*, 1981). In the production of antibodies, T and B lymphocytes play distinctive but interdependent roles and experimentally these cells can be separated on the basis of their characteristic surface membrane composition (Wigzell *et al.*, 1972; Wybran, 1979). Differences in carbohydrate residues between mouse T and B cells have been reported (Krusius *et al.*, 1979) but there is little comparative information on the surface carbohydrates of equivalent human lymphocyte populations, although unseparated cells have been more extensively studied (Presant & Kornfeld, 1972; Van Beek *et al.*, 1978; Reisner *et al.*, 1979).

An interesting approach to the analysis of cell surface saccharides is to evaluate their capacity for interaction with lectins. Lectins are cell agglutinins which bind to sugar residues of specific configuration and sequence (for review, see Goldstein &

Hayes, 1978). A large number of lectins have been described with an extensive range of sugar specificities. In the present study, we have used FITC-conjugated lectins in conjunction with flow cytometry to examine the lectin binding properties of human peripheral blood lymphocytes either as an unseparated population or after fractionation into T-cell and B-cell enriched subsets.

Materials and methods

Lectins

FITC-conjugated lectins were obtained from Miles Chemicals; the molar ratios of FITC: protein for each lectin were: FITC–concanavalin A, 2.14:1; FITC–*Lens culinaris* (lentil) lectin, 1.8:1.

Homogeneity of lectin and effects of conjugation with FITC

The lectins used were purified by affinity chromatography by the suppliers and we checked their homogeneity on polyacrylamide-gel electrophoresis. Cell agglutination properties were not affected by FITC conjugation. Routinely we further evaluated the influence of coupling lectins to FITC by measuring cell fluorescence at varying ratios of FITC–lectin:native lectin while maintaining a constant lectin concentration. In each case the plot of concentration versus cell fluorescence was linear, and passed through the origin, demonstrating that

Abbreviations used: FITC, fluorescein isothiocyanate; Ig, immunoglobulin.

the native and conjugated forms behaved as a single molecular species.

Donors

Heparinized blood samples (150 ml) were taken from six healthy donors (age 28–36 years) and from one patient with Hodgkin's disease. Lymphocytes were separated by centrifugation (1000 g, 20 min, 4°C) on lymphocyte separation medium (Flow Laboratories, Irvine, Scotland, U.K.). Mononuclear cells were taken from the interface with a Pasteur pipette, washed twice in medium and adherent cells were depleted by incubation (30 min at 37°C) in 75 cm² Corning culture flasks in RPMI medium + 10% (v/v) foetal calf serum. Non-adherent cells were washed once, resuspended in foetal calf serum and the concentration adjusted to 5×10^6 /ml. Cells were over 95% viable by Trypan Blue exclusion and >97% were lymphocytes.

Separation of lymphocyte populations

Cells were separated into T-cell enriched or T-cell depleted fractions by (a) formation of rosettes with sheep erythrocytes or (b) passage through glass bead columns coated with human IgG and horse anti-(human IgG).

(a) Sheep erythrocytes were washed three times in phosphate-buffered saline and resuspended in foetal calf serum (2×10^8 ml). Equal volumes of lymphocytes and washed sheep erythrocytes were mixed, centrifuged (100 g, 5 min, 4°C) and allowed to stand overnight at 4°C. Pellets were gently resuspended and the E-rosetting and non-rosetting cells were separated by centrifugation on lymphocyte separation medium. Lymphocytes were freed from erythrocytes by osmotic shock. Each population was washed a further three times in RPMI medium, resuspended in RPMI medium and counted.

(b) Lymphocytes with Ig or Fc receptors at their surface were removed as described by Wigzell *et al.* (1972) by using a column of acid-washed Degalan beads coated with human IgG (Sigma) and horse anti-(human IgG).

Composition of lymphocyte populations

Cells were monitored for the capacity to form rosettes with sheep erythrocytes at 4°C for 4 h with a lymphocyte:erythrocyte ratio of 1:40 in foetal calf serum (total E rosetting cells). The presence of receptors for the Fc portion of Ig (Fc) was measured as described by Dickler (1976) and cell surface Ig was detected by using FITC-labelled sheep anti-(human Ig).

Lectin binding assay

Separated lymphoid cell populations were adjusted to final concentrations of 2×10^6 cells/ml in phosphate-buffered saline containing 0.1% bovine serum albumin; all washings and incubations of cells

with lectins were carried out in this buffered albumin solution. Cells (10^5 in 50 μ l) were added to a series of microtitre plates and varying amounts of FITC-labelled lectins were added from stock solutions of lectins of concentration 0.4 mg/ml. Final volumes were made up to 150 μ l. All solutions and cell suspensions were cooled to 4°C before mixing cells and lectins. Incubations were carried out at 4°C for 30 min with occasional agitation. Kinetic experiments showed that 30 min was an adequate time for attainment of the equilibrium of bound:unbound lectin. The degree of specificity of lectin binding was determined by incubation with appropriate monosaccharide inhibitors. These sugar ligands were mixed with lectins for 5 min before addition of cells. After incubation, the contents of each microtitre well were suspended in 1 ml of buffer and cell fluorescence was measured by flow cytometry. Competition studies were also carried out in which native lectins were assessed for their ability either to inhibit the binding to cell surfaces of FITC-conjugated lectins (i.e. native lectins added first to cell suspensions followed by FITC-lectins) or to displace FITC-lectins already bound to the cell surface. Additional details are given in appropriate Figure legends. At 4°C, cell-bound FITC-lectins appeared to be located mainly at the cell surface, with minimal intracellular fluorescence as judged by fluorescence microscopy.

Flow cytometry

The cells were measured in a Biophysics Cytofluorograf 4800A interfaced to a desk top computer (Hewlett-Packard 9845A). This has been previously described (Blackledge *et al.*, 1980a,b) and allows for analysis of cell populations by using two-dimensional histograms. The mean fluorescence of the cell populations with lectin bound was estimated by using the expression:

$$\left(\sum_{i=1}^{N_c} i \times n_i / N_c \times \sum_{i=1}^{N_c} n_i \right) - \text{zero correction}$$

where N_c is the total number of channels and n_i is the number of cells in channel i (modified from Bohn, 1976).

Values are corrected to allow for variations in cell auto-fluorescence. When 'mean cell fluorescence' values for individual lectins are compared (see Figs. 1, 3, 4 and 5) the data were corrected for the specific fluorescence of the lectin (number of FITC residues per molecule). This allows a direct comparison of numbers of lectin binding sites per cell but we have not determined absolute values for numbers of sites.

Cell sorting

Cell sorting on the basis of lectin-derived cell fluorescence was carried out on a Becton Dickinson

FACS-IV cell sorter. Sorting gates were set to sort to left and right the lower fluorescent and upper fluorescent cells respectively. The results of sorting were checked by using conventional flow cytometry and by determining the proportion of E-rosetting cells in each population.

Results

Comparison of the binding of FITC-lentil lectin and FITC-concanavalin A to unseparated lymphocytes

Initial experiments with FITC-lentil lectin and FITC-concanavalin A determined lectin binding at 4°C to unseparated lymphocytes as a function of lectin concentration. There was a significantly higher concentration, particularly at low lectin concentrations, of surface-membrane-bound FITC-lentil lectin molecules compared with FITC-concanavalin A (Fig. 1). The vertical axis, 'mean cell fluorescence', in Fig. 1 (and in the similar types of graph in Figs. 3–6) is directly proportional to the number of bound FITC-conjugated lectin molecules (see the Materials and methods section). FITC-lentil lectin achieved near-saturation binding at a much smaller concentration of added lectin than did FITC-concanavalin A. Two populations of fluorescent cells were observed in the unseparated lympho-

cytes after binding of FITC-lentil lectin (Fig. 2a); the differences in cell fluorescence were independent of cell size. In contrast, the interaction of lymphocytes with FITC-concanavalin A yielded only one cell population of relatively low fluorescence (Fig. 2b). We have examined ten lectins, including those described in this paper, and FITC-lentil lectin was the only lectin which gave separable cell populations on the cytogram (M. Harding & J. T. Gallagher, unpublished work).

Lymphoid cells were then sorted on the basis of their FITC-lentil lectin cell fluorescence by using a fluorescence-activated cell sorting system with the sorting gates adjusted to discriminate between the high- and low-fluorescence subsets illustrated in Fig. 2(a). The two sorted populations were then evaluated for their rosette-forming ability with sheep erythrocytes. Notably, 77% of the low FITC-lentil lectin subset were E-rosette-positive whereas only 27% of the high FITC-lentil lectin subset formed E-rosettes. This experiment demonstrated that the low reactivity with FITC-lentil lectin was correlated with an enrichment of T-lymphocytes (E-rosetting cells) and raised the possibility that the high FITC-lentil lectin fluorescence population was enriched in B lymphocytes.

Analysis of separated lymphocytes

Cells separated into E-rosetting and non-rosetting subgroups were examined for their cell surface markers and for their lectin reactivity. B-lymphocytes, considered as surface-Ig bearing cells, were almost entirely localized in the non-rosetting cells (Table 1). The two cell populations bound different amounts of FITC-lentil lectin over a wide range of added lectin concentrations (Fig. 3a) with B-enriched cells consistently binding more FITC-lentil lectin. Conversely, lymphocytes which passed unretarded through an Ig/anti-Ig column (Degalan-

Fig. 1. Comparison of cell fluorescence with FITC-lentil lectin and FITC-concanavalin A as a function of lectin concentration

α-Methyl mannoside was used at a final concn. of 5 mM. Cells (10^5 in $150\mu\text{l}$ of phosphate-buffered saline) were incubated for 30 min at 4°C with the above lectin concentrations before measuring cell fluorescence. α-Methyl mannoside was added to the lectin before addition of cells. x, FITC-lentil lectin; Δ, FITC-lentil lectin and α-methyl mannoside; O, FITC-concanavalin A; ■, FITC-concanavalin A and α-methyl mannoside. With the scale used, this latter plot was at the baseline.

Table 1. Composition of lymphocyte populations

Lymphocytes from human peripheral blood were evaluated for surface markers and separated into E-rosetting and non-rosetting cells with sheep erythrocytes. Surface markers were then determined on the separated cells which were also re-evaluated for their E-rosetting capacity. The Table also shows marker analyses on lymphocytes which failed to bind to a Degalan column (Degalan-passed). Values are medians of six determinations.

Lymphocyte population	Proportion (%) of lymphocytes with markers		
	E-Rosettes	Fc	Surface Ig
Unseparated	60	14	15
E-rosetting	89	12	2
Non-rosetting	17	20	25
Degalan-passed	81	<1	4

Fig. 2. Two-dimensional histograms and cytograms of cell fluorescence with FITC-lentil lectin (a) and FITC-concanavalin A (b) of unseparated (upper panels), non-rosetting (middle panels) and E-rosetting (lower panels) lymphocytes

Cells (10^5 in $150\mu\text{l}$ of buffer) were incubated at 4°C for 30 min with $1\mu\text{g}$ of FITC-lentil lectin or $8\mu\text{g}$ of FITC-concanavalin A.

column) were depleted in cells bearing surface Ig (B lymphocytes) and Fc-receptors (Table 1) and gave an identical binding curve to the E-rosetting, T lymphocytes (Fig. 3a).

We have now studied six healthy donors and despite some variation in fluorescence values each individual yielded non-rosetting cells of higher reactivity with FITC-lentil lectin than their E-rosetting counterparts.

Specificity of lectin interaction

The binding of both FITC-lentil lectin and FITC-concanavalin A to E-rosetting and non-rosetting cells was markedly decreased (by 80–95%) by the monosaccharide inhibitor, α -methyl mannos-

ide (Fig. 1). The inhibitory effect of the sugar decreased slightly with increasing lectin concentrations. When cells were pre-incubated with native concanavalin A (Fig. 4) or native lentil lectin (Fig. 5) the binding of the corresponding FITC-conjugated lectins showed a concentration-dependent decrease. Inhibition was particularly marked when native lentil lectin was used to inhibit the binding of FITC-lentil lectin. By comparison, native concanavalin A was a less effective inhibitor of FITC-concanavalin A binding and this relates to our finding that, unlike the binding of FITC-lentil lectin (Fig. 3a), FITC-concanavalin A binding does not reach a plateau value but continues to show a slight increase with each increment of lectin concentration (Fig. 3b).

Fig. 3. Concentration-dependence of the binding of FITC-lentil lectin (left panel) and FITC-concanavalin A (right panel) to E-rosetting and non-rosetting lymphocytes

Incubation conditions were as in Fig. 1. Δ , Non-rosetting with FITC-lentil lectin; $\times$, E-rosetting with FITC-lentil lectin; $\bullet$, non-rosetting with FITC-concanavalin A; $\circ$, E-rosetting with FITC-concanavalin A.

This may be due to lectin-lectin interactions or to the exposure of small numbers of cryptic concanavalin A binding sites at high bound-lectin concentrations.

Individuality of lectin binding sites

Pre-bound FITC-lentil lectin could not be displaced from cell surfaces by subsequent addition of either native lentil lectin or native concanavalin A; pre-bound FITC-concanavalin A behaved similarly. Thus, the rate of spontaneous dissociation of bound lectin was negligible. Pre-bound native lentil lectin, although a good inhibitor of FITC-lentil lectin binding (Fig. 5) did not inhibit the binding of FITC-concanavalin A. However, pre-bound concanavalin A caused a partial inhibition of the binding of FITC-lentil lectin (Fig. 4). These results show that FITC-lentil lectin and FITC-concanavalin A bind to different sites on the lymphocyte surface but that an initial interaction with concanavalin A impairs the ability of FITC-lentil lectin to recognize complementary saccharide sequences.

Discussion

Lectins are valuable tools for examining the composition of cell surface saccharides *in situ*; lectin-binding data permit analysis at the molecular level of the expression of carbohydrate sequences, and some information on the biochemical organiza-

tion of lectin-reactive groups may be acquired from competition studies.

Probably the most important observation in this study was that FITC-lentil lectin identified two distinct populations in human peripheral blood lymphocytes which corresponded to weakly-fluorescent T-cells (E-rosetting group) and highly fluorescent non-T cells which were enriched in B lymphocytes (Figs. 2a and 3). Lentil lectin will bind to complex biantennate oligosaccharides and to certain forms of triantennate structures, but a key feature of lentil lectin reactivity is the requirement for a fucosyl residue on the GlcNAc which is in *N*-glycosidic linkage to asparagine in the protein core (Kornfield *et al.*, 1981). Our findings suggest that T and B lymphocytes differ strikingly in their expression of such sequences, to a degree which permits the sorting of these two populations on the basis of their FITC-lentil lectin fluorescence. To our knowledge, this is the first observation of differences in complex fucosylated saccharide chains on subsets of human peripheral blood lymphocytes and the use of this distinction for cell sorting represents a new and rapid method for enrichment of T and B cells without recourse to pre-treatment of the surface membrane with neuraminidase, an essential step in the fractionation of these cell populations with *Helix pomatia* lectin (Hellström *et al.*, 1976).

We did not anticipate that FITC-lentil lectin and FITC-concanavalin A would bind to different sites on the lymphoid surface, since binding of both

Fig. 4. Influence of native concanavalin A on the binding to lymphocytes of FITC-concanavalin A (8 µg) and FITC-lentil lectin (2 µg)

Cells (10^5 in 150 µl of buffer) were incubated for 15 min with various concentrations of native concanavalin A. FITC-conjugated lectins were added for a second 15 min incubation in a final volume of 200 µl. ▲, Non-rosetting with FITC-lentil lectin; ■, E-rosetting with FITC-lentil lectin; ○, non-rosetting with FITC-concanavalin A; ●, E-rosetting with FITC-concanavalin A.

lectins is inhibited by similar monosaccharides (Howard *et al.*, 1971; Allen *et al.*, 1976; see also Fig. 1). Like lentil lectin, concanavalin A will bind to complex biantennate chains but the interaction does not require a fucose residue (Narasimhan *et al.*, 1979; Baenziger & Fiete, 1979). Concanavalin A will bind strongly to oligomannosyl-type chains, in contrast to the weak interaction of FITC-lentil lectin with mannose-terminated sequences (Kornfeld *et al.*, 1981). Possibly these differences in affinity and specific sugar requirements are amplified within the complex microenvironment of the cell surface so that fucosylated sequences may only bind to lentil lectin and oligomannosyl chains to concanavalin A. It will be important to resolve the molecular basis of this distinction to appreciate fully the information

Fig. 5. Influence of native lentil lectin on the binding to lymphocytes of FITC-concanavalin A and FITC-lentil lectin

Experimental details were as in Fig. 4. ▲, Non-rosetting with FITC-lentil lectin; ■, E-rosetting with FITC-lentil lectin; ○, non-rosetting with FITC-concanavalin A; ●, E-rosetting with FITC-concanavalin A.

that may be derived from binding data with these lectins.

Topographically, the partial inhibition of FITC-lentil lectin binding by pre-exposure of lymphocytes to concanavalin A (Fig. 4) indicates that the blocked lentil lectin sites are very close to concanavalin A sites, the inhibition arising either through steric hindrance or a concanavalin A-induced alteration in saccharide conformation. Possibly some lentil lectin sites are located more deeply within surface associated structures than are concanavalin A sites, an arrangement which might also explain why pre-binding with lentil lectin does not impair the reactivity of FITC-concanavalin A (Fig. 5). Inhibition studies of this type may ultimately lead to the 'mapping' of membrane saccharide domains.

The physiological significance of differences in expression of membrane saccharides is unknown. However, it is noteworthy that in a study of the functional heterogeneity of E-rosetting lymphocytes, we have isolated cells with non-specific cytotoxic activity against cultured tumour cells on the basis of FITC-lentil lectin binding (Vose *et al.*,

1981). Others have shown an association between binding of *Vicia vellosa* lectin and allo-specific cytotoxic functions (Kimura *et al.*, 1979). Clearly lectins are valuable markers of lymphocyte heterogeneity and their use in conjunction with monoclonal antibody reagents should allow further correlations of saccharide expression and lymphocyte function.

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