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I and i antigens of human peripheral blood lymphocytes cocap with receptors for concanavalin A

(lymphocyte surface/lymphatic leukemia/monoclonal antibodies/blood group I,i function)

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ABSTRACT **Surface immunofluorescence experiments using a human anti-i and two anti-I antisera have been performed on human peripheral blood lymphocytes. These are known to contain cold-reactive monoclonal IgM antibodies against the carbohydrate sequence:**

Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Gal—(anti-i Den)

and the 1 → 4, 1 → 6 domain (anti-I Ma) and the 1 → 4, 1 → 3 domain (anti-I Step) of the branched I-active structure:

Galβ1 → 4GlcNAcβ1 ↘ 6 3 Galβ1 → 4GlcNAcβ1 → 3Gal—.
Galβ1 → 4GlcNAcβ1 ↗

A high proportion of B- and T-type lymphocytes express these I and i determinants. In the presence of anti-human immunoglobulin, the cold-reactive membrane-associated complexes of I-anti-I and i-anti-i become stabilized, and redistribution (with patching and capping) can be elicited at 37°C. Dual fluorescence experiments have shown striking concordant staining of I or i (fluorescein) caps and patches with concanavalin A (rhodamine) reactive sites on normal and leukemic cells, suggesting that a proportion of I and i active structures of lymphocyte membranes are structurally associated or physiologically coupled with glycoproteins carrying oligosaccharides with branched mannosyl cores.

The I and i antigens are best known as surface antigens of human adult and fetal erythrocytes, respectively (1). They are recognized by human autoantibodies, most of which are cold-reactive monoclonal IgM proteins (2). However, these antigens are not confined to human erythrocytes; they have been detected on human lymphocytes, polymorphonuclear leukocytes, macrophages (3), and various cultured cells (4, 5) and in water-soluble form in certain secretions (2). Moreover, they are variably expressed on erythrocytes (6) and cultured cell lines of several animal species (7, 8).

The molecular basis of I and i specificities has been elucidated recently (9–11): i activity has been shown to reside on the straight oligosaccharide chain

Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Gal—.

The addition of a Galβ1 → 4GlcNAcβ1 → 6 chain to the middle galactose converts it into a branched I-active structure on which various monoclonal anti-I antibodies recognize different oligosaccharide domains. Both of these structures serve as precursor chains for the blood group ABH antigens (12, 13) and for certain erythrocyte gangliosides (14) but their I and i activities are totally (in ABH antigens) or partially (in gangliosides) masked by these additional external substitutions.

The Ii antigens are not only of pathological interest as targets of autoantibodies in cold hemagglutinin disease (15) in man but they are also of considerable biological interest because of (*i*) the developmental changes—i to I in the first year of life—on human erythrocytes (1), (*ii*) the tumor-associated increase in expression of I in certain adenocarcinomas (16), and (*iii*) the cell cycle-associated changes in i expression on lymphocytes (5). Considerable information is accumulating on the Ii active structures of erythrocyte membranes; these include band 3 protein (17), gangliosides (14), and polyglycosyl ceramides (18). Their antigenic change from i to I is believed to be associated with increased branching of carbohydrate chains (10). The increased I antigen expression in adenocarcinomas of blood group ABH-secreting tissues is almost certainly associated with incomplete terminal glycosylations with accumulation of precursor chains (19). Far less is known about the Ii active structures of lymphocytes.

In the present communication, dual-label surface fluorescence studies have shown that I- and i-active structures cocap with concanavalin A (Con A) receptors on peripheral blood lymphocytes. These observations suggest that a substantial proportion of the I and i activity is carried on or functionally associated with lymphocyte membrane proteins bearing oligosaccharides with the branched mannosyl cores (20) that are recognition sites for Con A.

MATERIALS AND METHODS

Anti-I, Anti-i, and Control Sera. Two anti-I antisera (Ma and Step) of different fine specificities and an anti-i serum (Den) were used in immunofluorescence experiments at dilutions of 1:100 (Ma) and 1:700 (Step and Den). These antisera were obtained from patients with chronic cold hemagglutinin disease and have been shown to contain monoclonal IgM κ (Ma and Step) or IgM λ (Den) antibodies (21, 22). Anti-i Den recognizes the straight oligosaccharide sequence (see ref. 9):

Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Gal—.

Ma recognizes the Galβ1→4GlcNAc1→6 chain (12) whose substitution onto the i-active sequence converts it into the I-active structure (see refs. 10 and 11):

Galβ1 → 4GlcNAcβ1 ↘ 6 3 Galβ1 → 4GlcNAcβ1 → 3Gal—.
Galβ1 → 4GlcNAcβ1 ↗

Step reacts best with the 1→4, 1→3 component of this branched I-active structure (10, 11).

As a control, serum from a healthy donor (T.F.) was used to which 4 mg of a Waldenström macroglobulin [IgM λ (Ham)] was added per ml; this serum was used at 1:100 dilution.

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Abbreviations: Con A, concanavalin A; RCon A, rhodamine-conjugated Con A.

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Con A. Rhodamine-conjugated Con A (RCon A) was purchased from Vector Laboratories (Norato, CA). Rhodamine conjugation was found not to affect dose–response curves or optimal concentrations for blastogenic effects (5 μg/ml) (unpublished observations). RCon A binding could be readily visualized on peripheral blood lymphocytes when 10^6 cells were incubated with 1 μg of the lectin per ml for 30 min; patch and cap formation were optimal at 20–30 μg/ml. Higher concentrations produced progressively greater agglutination of cells and were unsuitable for immunofluorescence studies.

Lymphocytes. Peripheral blood lymphocytes were isolated, by using a Ficoll/Triosil gradient, from six normal volunteers, one patient with acute prolymphocytic leukemia (T-cell type with surface Ig-negative, sheep erythrocyte-rosette-forming cells), and one patient with chronic lymphocytic leukemia. Both of the leukemic patients had peripheral leukocyte counts greater than 1×10^5 per μl. Defibrinated blood was diluted 1:3 with phosphate-buffered saline at pH 7.4, and 8 ml was layered on 3 ml of Ficoll/Triosil, (specific gravity, 1.077) and centrifuged at $800 \times g$ for 25 min. The mononuclear cell layer was washed thrice with RPMI-1640 medium and adjusted to 10^6 cells per 100 μl of medium for staining. For B-cell enrichment, $4–6 \times 10^7$ cells at a concentration of 2.5×10^7 cells per ml of medium were incubated with neuraminidase-treated sheep erythrocytes applied to a Ficoll/Mertrizoate gradient (specific gravity, 1.090) and centrifuged for 25 min at $1000 \times g$. The nonrosetted mononuclear cell layer was harvested, washed, cultured for 30 min in RPMI-1640 with 10% fetal calf serum to remove adherent cells, washed, and adjusted to 10^6 cells per 100 μl of medium.

After the rosetting procedure, 70% of the cells were positive for surface immunoglobulin as shown by staining with a polyvalent anti-human Ig reagent (see below) conjugated with fluorescein.

Immunofluorescence. Surface staining was performed as described (23). Lymphocytes were incubated with RPMI-1640 medium, control serum, or anti-I or anti-i antiserum for 30 min at 4°C, washed twice at 4°C with medium, and incubated at 4°C with fluorescein-conjugated $F(ab')_2$ fragments of a goat antiserum specific for human γ, μ, κ, and λ chains (23). For dual-label studies, washed stained cells (10^6/ml medium) were patched or capped by incubating for 0, 10, 20, 30, or 60 min at 37°C; they were then incubated with RCon A at 20 or 30 μg/ml for 30 min at 4°C and washed twice at 4°C. Alternatively, RCon A staining and capping were done first, followed by I,i staining at 4°C. Cell viability at the conclusion of each experiment was found to be >95% as determined by exclusion of 0.1% trypan blue. The stained cells (2.5×10^5 cells per slide) were cytocentrifuged (Shandon Instruments) at 700 rpm for 7 min. In each dual-label experiment, 100 cells were counted for fluorescein/rhodamine fluorescence and for concordant or discordant staining patterns by using a Zeiss fluorescent microscope with Plöem epi-illumination and phase-contrast microscopy.

RESULTS

Immunofluorescence Control Experiments. In control experiments to demonstrate immunofluorescence associated with surface Ig, lymphocytes were stained with fluorescein-conjugated $F(ab')_2$ fragments of polyvalent anti-human Ig after incubation with culture medium or control serum. The proportions of cells stained were: 16–43% with unfractionated peripheral blood lymphocytes, 60% or greater with B-enriched lymphocytes, 77% with the chronic leukemia cells, and 0% with acute prolymphocytic leukemia cells. In previous studies with the same anti-human Ig reagent, positive staining was obtained with 11.5 ± 2.5 (mean ± SD)% of normal peripheral blood lymphocytes from 30 normal donors (23). The higher percentages of stained cells in the present experiments in part reflect selection of donors with known high B-cell counts. In addition, Fc receptor binding during incubation with control serum was not ruled out.

Single Immunofluorescence and Capping Experiments with Anti-i and Anti-I Antisera. With normal peripheral blood lymphocytes, 90% of cells showed strong surface staining with anti-i Den; there was diffuse peripheral staining and coarse patching at 4°C. This staining pattern was clearly distinguishable from the finely stippled staining due to surface Ig. Similar staining patterns were observed with anti-I Ma and Step but the overall intensity was less than with the anti-i antiserum and there was considerable variation in intensity of staining from cell to cell; there was also variation in the proportion of cells stained from donor to donor, ranging from 56 to 98% with anti-I Step and from 36 to 75% with anti-I Ma. Of the B-enriched cells, 90% or more were stained with the three antisera, and the pattern and intensity of fluorescence were again clearly distinguishable from those of surface Ig staining.

Exploratory experiments with normal lymphocytes showed that redistribution of I and i fluorescence and cap formation could be readily achieved by incubation for 10 min at 37°C; at 30 min there occurred moderate shedding of fluorescent aggregates, presumably due to the dissociation of anti-I and anti-i antibodies from their receptors. Subsequent capping experiments were therefore performed for 10 min at 37°C and terminated by immediate cytocentrifugation. This short incubation time had the advantage of causing relatively little redistribution of the surface Ig on B lymphocytes.

The chronic lymphocytic leukemia cells showed a decreased proportion, relative to normal lymphocytes, stained with anti-i Den (70% compared to 93–100%); on the other hand, the proportion and intensity of staining with anti-I Ma were increased (100% compared to 40–75%). The acute prolymphocytic leukemia cells stained well with all three cold agglutinin antisera. There was excellent I and i patch and cap formation with cells from both leukemic patients.

Dual-Label Fluorescence with Anti-i or Anti-I (Fluorescein) and Con A (Rhodamine). There was concordant staining of a high proportion of patches and caps formed with anti-I or anti-i and Con A. The coredistribution was noted with unfractionated peripheral blood lymphocytes from six donors as well as with the B-enriched, the chronic lymphocytic leukemia, and the acute prolymphocytic leukemia cells (Table 1). The concordant staining pattern was noted under all staining options: (*i*) I or i staining and capping followed by Con A staining at 4°C (summarized in Table 1); (*ii*) Con A staining and capping followed by I or i staining at 4°C (data not shown). The coincident staining was most pronounced under option *i* with which there was only a minor amount of Con A staining at sites negative for fluorescein (I,i) (Fig. 1). Under option *ii* the concordance within individual cells was variable—i.e., the proportion of Con A patches showing concordant I,i staining varied and often there was some I,i staining at sites negative with Con A.

DISCUSSION

A high proportion of normal unfractionated and B-enriched peripheral blood lymphocytes and B- and T-cell type leukemia cells showed fluorescent staining with the anti-I and anti-i antisera. This is in agreement with previous lymphocytotoxicity (24) and antibody binding (25) experiments that indicated that human B and T lymphyoctes express both I and i antigens. The diminished staining with anti-i Den and increased staining with

Table 1. Dual-label fluorescence of peripheral blood lymphocytes with anti-I, anti-i or control sera (fluorescein) and Con A (rhodamine)

Cells	% of cells stained*			
	F	R	F + R	Concordant†
Peripheral blood lymphocytes:‡				
Medium or serum controls	0–15	63–90	18– 43	0– 5
Anti-i Den	0– 6	0	93–100	91–100
Anti-I Step	0–10	0–40	56– 98	98§
Anti-I Ma	0–10	16–60	36– 75	61§
B-Enriched lymphocytes:¶				
Medium or serum control	0	45	60	0
Anti-i Den	0	0	100	100
Anti-I Step	1	2	96	96
Anti-I Ma	0	3	90	90
Chronic lymphocytic leukemia:¶				
Medium or serum control	0	24	77	0
Anti-i Den	0	30	70	70
Anti-I Step	0	9‖	91‖	86‖
Anti-I Ma	0	0	100‖	100‖
Prolymphocytic leukemia:¶				
Medium or serum control	0	100	0	0
Anti-i Den	0	0	100	100
Anti-I Step	0	0	100	100
Anti-I Ma	0	0	100	100

* F, with fluorescein only; R, with rhodamine only; F + R, with both fluorochromes. The data shown for healthy donors are the range observed in the individuals tested.

† Concordant = percentage of cells showing coincident patching or capping of fluorescein and rhodamine after redistribution of I,i antigens.

‡ Six healthy donors were tested with serum controls and anti-i Den; four donors were tested with anti-I Step and Ma. I,i staining was first carried out at 4°C; after warming to 37°C for 10 min, Con A staining was performed at 4°C.

§ The majority of F + R positive cells showed concordant capping with all four donors; however, formal counting of concordant cells was performed with one donor only.

¶ One donor.

‖ Less than 100 cells counted.

anti-I Ma of the chronic lymphocytic leukemia cells (relative to normal lymphocytes) is also in agreement with previous data (3, 24).

The human monoclonal anti-I and anti-i antibodies are powerful reagents recognizing defined carbohydrate chains on cell surfaces. However, their rapid dissociation from their antigens at 37°C imposes limitations on their use in biological experiments. However, the present studies have shown that, in the presence of a second antibody (anti-human Ig), the I–anti-I and i–anti-i complexes at the cell surface become stabilized. Thus, redistribution (capping) experiments at 37°C can be performed. The I,i fluorescence was very bright, and the capping and patching of these antigens occurred more rapidly than the capping of surface Ig of B lymphocytes; therefore, I,i caps could be easily distinguished from the Ig fluorescence after 10 min of warming.

Concordant redistribution of Con A receptors with I and i antigens was observed with normal and leukemic peripheral blood lymphocytes, especially when the I,i antigens were redistributed first. This suggests that a high proportion of Con A-reactive structures are closely associated with I,i-active structures on lymphocyte membranes, and the possibility is raised that a substantial proportion of Ii structures† are carried on glycoproteins bearing branched mannosyl cores which are reactive with Con A (20). The I and i antigens have previously been shown to occur on glycoproteins (mucins) with *O*-glycosidically linked carbohydrate chains (12) and on glycosphingolipids and gangliosides of erythrocyte membranes (9, 10, 14) which are usually devoid of oligosaccharide chains with mannosyl core structures. The first indication that they may also be carried on glycoproteins with oligomannosyl structures came from affinity chromatography experiments in which purified anti-I and anti-i antibodies were used as adsorbents to isolate the I- and i-active components from pepsin-digested human amniotic fluid (2). Small amounts of mannose were regularly found in the eluted antigen preparations.

In addition, structural studies of glycopeptides of bovine thymus plasma membranes (26) have demonstrated the occurrence of oligosaccharide chains, with branched mannosyl cores, containing the terminal oligosaccharide sequence Galβ1→4GlcNAcβ1→6 Man—. It would be predicted that this sequence would react with anti-I Ma. Also, a subpopulation of band 3 protein of erythrocyte membranes, whose carbohydrate components are mannose-containing oligosaccharides *N*-glycosidically linked to protein, carries blood group I activity (17). Moreover, polyglycosyl peptides have recently been isolated from Pronase-digested erythrocyte membranes (27) and these contain highly branched oligosaccharides with mannosyl cores and terminal branched sequences known to carry blood group I activity. An additional possibility is that the I,i-active and Con A-active structures are on separate oligosaccharide chains carried by the same glycoprotein molecules. The finding of partial concordance of I,i and Con A staining within individual cells after redistribution of Con A receptors indicates that not all of the molecules are associated. Such partial concordance of I,i and Con A receptors was recently observed with the human erythroblast cell line K562 which would be predicated

† Cocapping of I,i-active and Con A-active structures is not a general phenomenon on all cell types. For example, in cultured cell lines from several animal species we have observed extensive or limited concordance in some and total discordance in others (8).

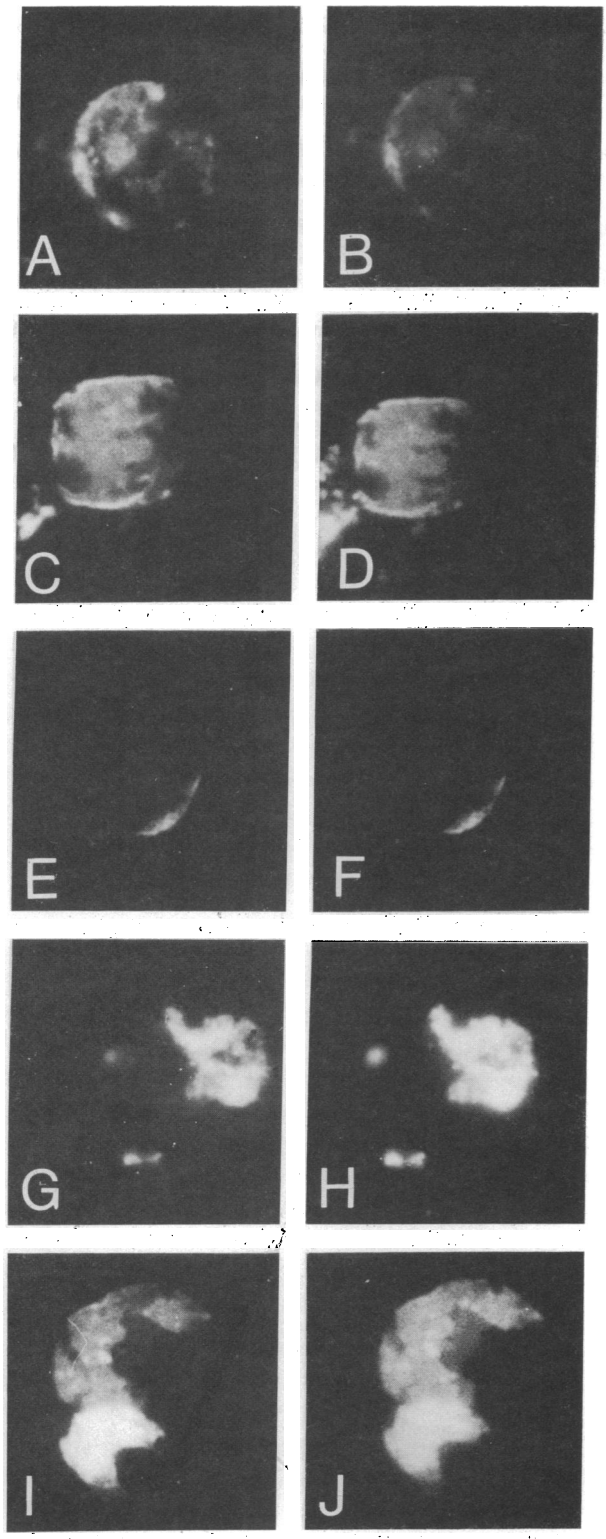

FIG. 1. Dual-label immunofluorescence of normal peripheral blood lymphocytes for i antigen using anti-i Den (fluorescein) (*Left*) and Con A receptors (rhodamine) (*Right*). Experimental conditions were as described in Table 1. Striking concordant distribution of i and Con A binding sites are shown in single cells (*A–H*) and in two adjacent cells (*I* and *J*). Three types of redistribution are shown: patching (*A/B; C/D*), capping (*E/F; I/J*); and capping with uropod formation (*G/H*). (×3375.)

to contain both glycoprotein and glycolipid components with i activity (8).

Among the numerous lymphocyte glycoproteins that bind Con A are the major histocompatibility antigens. Preliminary dual-label experiments have shown that redistribution of the major histocompatibility antigens of peripheral blood lymphocytes with (*i*) sheep antiserum to β_2-microglobulin, (*ii*) mouse hybridoma antibody (W6/32) to HLA-AB common antigens (28), and (*iii*) mouse hybridoma antibody (DA2) to HLA-DRW common antigens (provided by Michael Crumpton) results in a considerable degree of concordant redistribution of i antigen staining with anti-i Den (unpublished observations). Thus, the possibility is raised that (*i*) a subpopulation of the major histocompatibility antigens, like band 3 protein of erythrocytes, carries I,i antigens or (*ii*) the I,i- and Con A-active membrane components are part of a physiologically coupled transmembrane receptor–cytoskeletal system and automatically cocap. Affinity chromatography experiments with insolubilized anti-I,i and anti-Con A adsorbents and solubilized lymphocyte membranes should now be performed in order to investigate directly the biochemical basis of this dual reactivity.

The availability of monoclonal anti-I and anti-i antibodies and the ability to perform specific redistribution experiments now also opens the way to systematic investigations of the biological functions of I,i-active molecules of cell membranes. There are already indications that the I,i-active molecules may have roles as first messengers on lymphocyte membranes because, in separate immunofluorescence experiments (to be described in detail elsewhere), we have observed coincident distribution of cyclic GMP and cyclic GMP kinase in concordance with I and i patches and caps in peripheral blood lymphocytes of man.

The wide distribution of I,i-active structures (8), their apparent association with Con A-binding regions of several cell types, the binding activity of I,i structures with an endogenous lectin (29), and the demonstration of associated redistribution of cyclic GMP and its related kinase which are considered to be involved with lymphocyte activation (30) suggest important biological roles for the I,i oligosaccharides as surface structures whose perturbations are communicated to the interior of cells.

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