

The V4-34 Encoded Anti-i Autoantibodies Recognize a Large Subset of Human and Mouse B-Cells

Submitted on July 2, 1996

(communicated by Peter C. Nowell, M.D., 07/10/96)

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ABSTRACT: Autoantibodies to the i, I and Pr₂ carbohydrate determinants bind red blood cells, preferentially at low temperature in vitro. Using multiparameter flow cytometric analyses, we demonstrate that each of these autoantibodies also react with human and mouse lymphocytes at physiologic temperatures. The anti-Pr₂ autoantibody recognizes a glycoprotein determinant(s) expressed by a subset of both T and B lymphocytes. In contrast, the binding of anti-i and anti-I antibodies each is restricted to B-lymphocytes. The anti-i autoantibody binds to over 50% of all B cells, whereas the anti-I antibody reacts with less than 10% of either tonsillar or blood B cells. Prior studies identified that the B cell isoform of CD45 (B220) has the linear poly-N-acetyllactosamine that forms the "i" determinant. Because anti-B220 antibodies recently have been reported to influence T-dependent B-cell isotype switching, we tested each antibody for its ability to influence the production of secondary Ig isotypes by murine splenocytes co-cultured with a stimulator helper T cell clone. We find that addition of anti-i antibody increases the proportion of B cells secreting secondary Ig isotypes. In contrast, the anti-I antibody had no such effect. These findings imply that stimulation of B cells through the highly conserved carbohydrate determinant that forms the "i" antigen may be of physiologic importance in T-dependent B-cell differentiation.

Keywords: cold agglutinins, murine and human, B lymphocytes, V_H4-34 gene

INTRODUCTION

Previously, we investigated anti-B cell autoantibodies from patients with Wiskott-Aldrich syndrome, a rare X-linked immunodeficiency disorder (1). By flow cytometric analysis, each of these autoantibodies was found specific for a carbohydrate-dependent epitope expressed by a subset of human B cells inclusive of most B cells that co-express the CD5 surface antigen. Molecular analyses of different clones of IgM_κ or IgM_λ autoantibody-producing B cells revealed that

each expressed V4-34 (V_H4.21), a highly conserved Ig heavy chain variable region gene (V_H gene) that frequently is found to encode monoclonal anti-i/I autoantibodies (2,3,4,5). Indeed, we found that each antibody was specific for the "i" antigen present on neonatal erythrocytes, a determinant often recognized by monoclonal anti-i cold agglutinins. Because these anti-B cell autoantibodies were similar to previously defined anti-i autoantibodies, this study implied that cold agglutinins of persons without Wiskott-Aldrich syndrome also may react with

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mononuclear cell subsets.

Cold agglutinins are known as autoantibodies that bind carbohydrate determinants of red blood cells, preferentially at low temperatures. The first and most common carbohydrate specificity involves the related *i* and *I* antigens that can be detected on the red blood cells of multiple species (6). The *i* antigen is formed by a straight oligosaccharide chain of N-acetyllactosamine subunits attached to either ceramide or protein. The *I* antigen is formed when a branching enzyme attaches a β 1-6 branch to a galactose residue of the non-branched, *i* carbohydrate chain. The proportion of *i* to *I* antigenic structures on human red blood cells changes during development. Fetal and newborn red blood cells express mostly the non-branched *i* antigen, whereas adult red blood cells express mostly *I* antigen.

The second category of cold-agglutinins contains the *Pr* antigens. The *Pr* antigens are O-linked oligosaccharides located on the α and δ sialoglycoproteins (7,8). In contrast to the *I/i* antigens, the *Pr* antigens are destroyed by proteases, such as papain or ficin, or by neuraminidase, the latter indicating that sialic acid contributes to the epitope recognized by anti-*Pr* autoantibodies. The *Pr* antigen sub-group is determined by linkage of the terminal sialic acid residue to the oligosaccharide chain. For example, the *Pr*₂ antigen contains an α , 2-6 linkage (7,9), whereas the *Pr*₃ antigen is characterized by an α , 2-3 linkage. Furthermore, unlike the *I/i* antigens, the *Pr* antigens are expressed at high levels on both fetal and adult red blood cells.

In view of the anti-*i* reactivity of the anti-B cell autoantibodies of Wiskott-Aldrich syndrome patients, we investigated well characterized monoclonal cold agglutinins specific for *i*, *I*, or *Pr* determinants for their ability to bind human and mouse lymphocytes. Conceivably, each of the different major cold agglutinins recognize carbohydrate determinants that also are found on lymphocyte surface glycoproteins that are expressed differentially by distinct lymphocyte subsets. For this reason, we examined the lymphocyte subset specificity of each of these antibodies.

We also examined whether anti-*i/I* autoantibodies can influence lymphocyte function. The glycosylated 220 kDa B-cell isoform of CD45 has been noted to possess the straight oligosaccharide chains of N-acetyllactosamine that form the *i* determinant (10,11). Because antibodies specific for the B cell isoform of CD45 have been noted to have enhanced T cell-dependent B cell isotype switching (12), we examined whether anti-*i* or anti-*I* also could influence the production of secondary Ig isotypes *in vitro*. Collectively, our studies indicate that cold agglutinins should not be considered as being anti-erythrocyte autoantibodies, as each specifically binds to discrete lymphocyte subpopulations. Moreover, our studies reveal that such binding activity may have physiologic significance in cognate cell-cell interactions leading to lymphocyte differentiation.

MATERIALS AND METHODS

Cold Agglutinins

The monoclonal autoantibodies used in the present study are derived from EBV transformed clonal cell lines from patients with monoclonal cold agglutinin disease, as described (5,13). Clone 15A produces anti-*i*, clone 20A produces anti-*I* and clone LS 2 produces anti-*Pr*₂ antibodies. The IgM autoantibodies were purified from culture supernatant and from the heat eluate by passage over anti-human IgM (μ chain-specific)-CNBr-activated Sepharose columns (Cooper Biomedical, Malvern, PA; Pharmacia, Piscataway, NJ). The bound proteins were eluted with 0.1 M glycine, pH 2.5, rapidly neutralized, and then dialyzed against PBS. The antibodies were biotinylated with Normal-Human-Serum-Biotin (NHS-biotin, Pierce, Rockford, IL), according to the manufacturer's instructions. The biotinylated autoantibodies were dialyzed and concentrated with a collodium bag apparatus (Schleicher & Schuell, Keene, NH.) and tested for their ability to agglutinate red blood cells. Each of the purified antibodies was shown not to induce cytotoxicity when incubated with lymphocytes at either 4°C or

37°C for 30 minutes. The antibodies were used in a concentration of 2 µg/λ (which was also used in the flow cytometry experiments). In all instances, the viability of the cells cultured or stained with any of the IgM antibodies remained above 90%.

Immunofluorescence Studies

Lymphocytes were isolated from mouse spleen and human tonsil by gentle teasing of the tissue followed by Ficoll-Hypaque (Organon Teknika, West Chester, PA and Cedarlane Laboratories, Hornby, ON, Canada) gradient centrifugation. Human PBL were obtained from healthy donors by phlebotomy and also isolated by Ficoll-Hypaque gradient centrifugation. Cell preparations were incubated at 4°C with the appropriate dilution of fluorochrome-conjugated monoclonal antibodies specific for CD19, CD20, CD22, CD23, CD5, CD3 or CD45RA (from Becton Dickinson, Mountain View, CA). Anti-IgD is an IgG₁ anti-human δ heavy chain (DA4-1) from the American Type Tissue Collection (Rockville, MD). Cell surface binding of b-anti-i, b-anti-I and b-anti-Pr₂ was detected by second step staining with avidin coupled to either fluorescein (FITC-avidin, Molecular Probes, Eugene, OR), phycoerythrin (PE-avidin, Becton Dickinson) or Texas Red (TR-avidin, Molecular Probes) for two or three color analyses. For the murine studies, fluorochrome conjugated anti-Ly-1 (53-7.3), anti-B220 (RA3-6B2) and anti-Thy-1 (G7) were obtained from Pharmingen (San Diego, CA). Cells were analyzed with a FACScan (Becton Dickinson) equipped with Lysis II software. To determine the thermal amplitude of autoantibodies, the autoantibodies were incubated with lymphocytes at 4°C, 25°C, and at 37°C, while all subsequent washing steps were carried out at 4°C.

Neuraminidase Treatment of Lymphocytes and Red Blood Cells

Lymphocytes (10⁷ cells), isolated from peripheral blood by Ficoll-Hypaque were

incubated in 1 ml of RPMI 1640 media containing 1 unit of freshly added *V. Cholera* neuraminidase (Sigma, St. Louis, MO). After one hour incubation at 37°C, the lymphocytes were washed twice in PBS prior to FACS analysis (14). As a control, we treated red blood cells in a similar fashion and documented the effect of neuraminidase by its inhibition of agglutination of red blood cells with the anti-Pr₂ antibody.

Microculture Studies

A detailed description of the reagents and conditions for the microcultures containing limiting numbers of B cells and non-limiting numbers of T cells and dendritic cells (DC) has been recently published (12).

B cells. PP cells were isolated as described (15) from (C57BL/6 x C3HeB/FeJ)F₁ mice. Adherent cells were depleted by incubation of the cell suspension on plastic petri dishes for 2h at 37°C. For depletion of T cells, the cells were suspended at 1 x 10⁷ cells/ml in 1/500 dilution of anti-Thy1.2 ascites fluid (Cedarlane Laboratories, Hornby, ON, Canada). Viable cells were isolated on Ficoll-Paque (Pharmacia, Piscataway, NJ) and found to be >95% pure B cells by staining for surface Ig and MHC class II.

T cells. The Th cell clone D10.G.4.1 (D10, (16) TIB224) was obtained from American Type Culture Collection and maintained by alternate weekly cycles of stimulation and rest in Click's medium (Irvine Scientific, Santa Anna, CA) supplemented with 10% FCS (GIBCO, Grand Island, NY), 2 mM L-glutamine (GIBCO) 10⁻² mM β-mercaptoethanol (Sigma), and 50 µg/ml of Gentamycin (GIBCO). Resting cells (2 x 10⁴ /ml) were stimulated with 100 µg/ml of Conalbumin, irradiated C3H spleen cells (5 x 10⁵ /ml), and 5% rat Con A supernatant (prepared from rat spleen cells stimulated for 48 h with Con A-Sepharose beads). After 7 days, cells were harvested and either rested for 1 wk in medium supplemented with 5% Con A supernatant for propagation of the

line, or rested for 48 h in medium alone for use in microcultures. D10 cells are specific for Conalbumin presented in the context of I-A^k and are alloreactive for cells bearing I-A^b (17-18). Allostimulation of I-A^b bearing B cells by D10 cells in clonal microcultures is haplotype restricted (19).

DC. DC (dendritic cells) were isolated from PP of C3HeB/FeJ mice (19,20). We took advantage of the haplotype restriction of D10 cells by preparing DC from a strain that cannot stimulate D10. Controls in each experiment included cultures of DC and D10 cells receiving all of the various treatments but lacking B cells. Microcultures were established in 60-well Terasaki plates (Nunc, Intermountain Scientific, Bountiful, UT) and incubated in 5% CO₂ at 37°C. After 9 days, supernatants were harvested into 200 µl of medium and 30 µl was used for each ELISA.

ELISAs. To identify IgA in the well supernatants, affinity purified goat anti-mouse Ig (Fab fragments) was used to coat polystyrene microtiter plates and biotinylated monoclonal goat anti-mouse IgA for detection. To identify IgG₁ in the well supernatants, affinity purified rat anti-mouse IgG (Axell) was used to coat polystyrene microtiter plates and biotinylated monoclonal rat anti-mouse IgG₁ (Zymed, San Francisco, CA) for detection. The biotinylated reagents bound were detected by horseradish peroxidase-avidin (Vector Laboratories, Burlingame, CA). ABTS (Sigma) and H₂O₂ were used to develop the plates, and absorbance was read at 410 nm in a Dynatech ELISA reader (Dynatech, Chantilly, VA). For ELISAs, appropriate myeloma proteins were used to generate standard curves in each experiment. The assays were sensitive to 1 ng/ml for IgM/G/A and to 0.1 ng/ml for IgE. Positive wells were at least fourfold above these cutoffs.

Statistical analyses. The level of significance was estimated using the χ^2 test.

RESULTS

Reactivity of Anti-i, I and Pr₂ Autoantibodies With Human Tonsillar and Blood Lymphocytes

Anti-i/i binding. Multiparameter FACS analyses showed that both the anti-i autoantibody, designated 15A, and the anti-I autoantibody, designated 20A, bind to a subpopulation of B cells. As shown in figures 1 and 2, only CD19⁺ B cells are stained by 15A or 20A (representative of six staining experiments). While 15A stains approximately 50-60% of CD19⁺ B cells of either human tonsil or blood, 20A is only weakly reactive, staining 6-9% of the B cells. 15A preferentially reacted with tonsillar B cells that express surface IgD, displaying a reactivity pattern for tonsillar B cells similar to that of HY18, an IgM_κ anti-B cell autoantibody produced by a B cell clone from a patient with Wiskott-Aldrich syndrome (1). In addition, multiparameter FACS analyses indicate that most B cells that express CD5 also react with 15A, as noted previously for HY18 (1). Finally, the binding of biotinylated HY18 (anti-i) to B cells could be inhibited by 50% by prior incubation of the lymphocytes with the purified non-conjugated 15A (anti-i), while purified human IgM (0.1 mg/ml) of irrelevant specificity neither reacted with human lymphocytes nor competed with HY18 or 15A for binding to human B cells (data not shown). Thus, these experiments indicate that HY18 and 15A, both of which have relative anti-i specificity by RBC agglutination, also recognize similar determinants on lymphocytes. The carbohydrate specificity, e.g. anti-i was further substantiated by demonstrating that the antibody reactivity was abolished by prior treatment of red blood cells and/or lymphocytes with β -galactosidase (1).

Anti-Pr₂ binding. The biotinylated anti-Pr₂ autoantibody, bound to 70-80% of both B and T lymphocytes (figure 3). Similar results were observed in four separate experiments of different samples of peripheral blood lymphocytes. Thus, unlike the anti-i/I autoantibodies, the anti-Pr₂ antibody binds to both B and T cells.

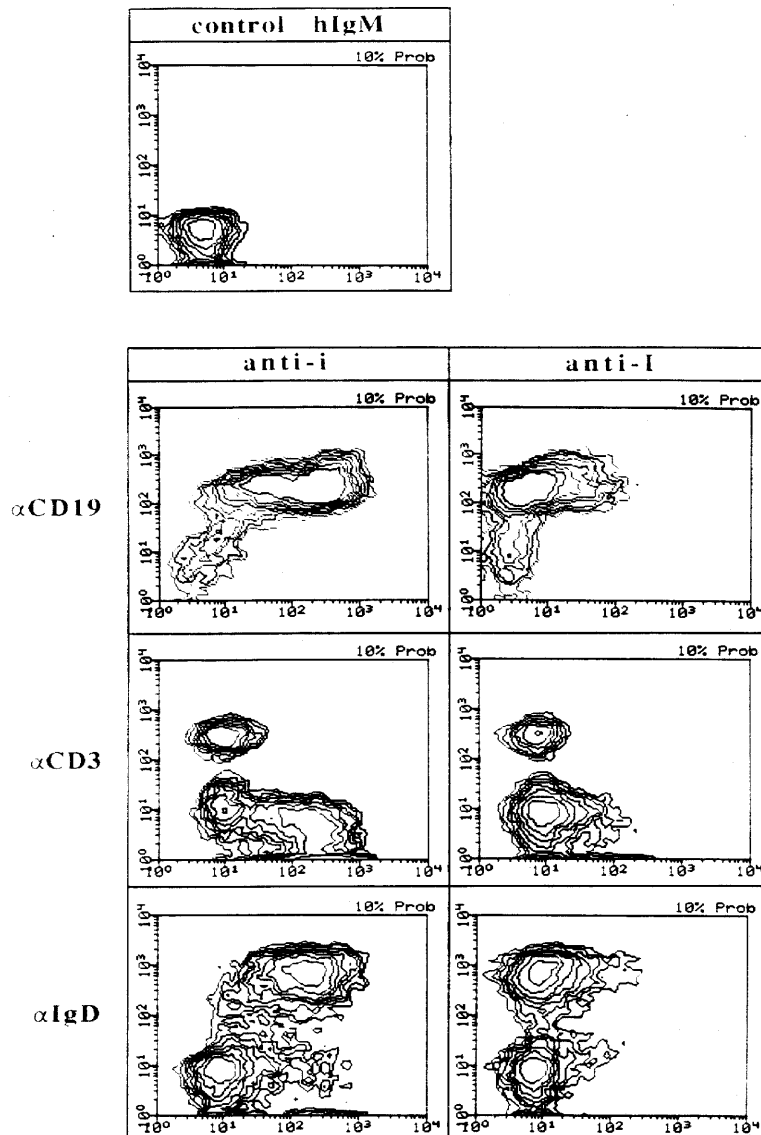


Figure 1. Multiparameter flow cytometric analysis of human tonsillar lymphocytes to determine the fractions of lymphocyte subset(s) that react with anti-i or anti-I autoantibodies. Lymphocytes were reacted with antibody 15A-biotin/avidin-FITC (anti-i) and 20A-biotin/avidin-FITC (anti-I) and with PE conjugated antibodies specific for CD19, CD3 and IgD reagents. The top panel represents lymphocytes stained with a control biotinylated human IgM of irrelevant specificity and avidin-FITC. Plots show levels of expression by use of 10% probability contour lines.

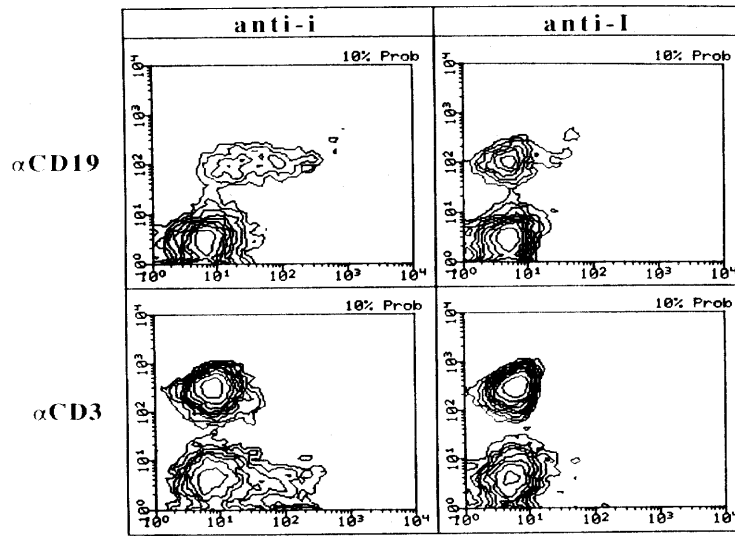


Figure 2. Flow cytometry of human peripheral blood lymphocytes. Two color combinations of reagents are used to determine the fractions of anti-i positive and anti-I positive lymphocytes that co-express CD19 or CD3.

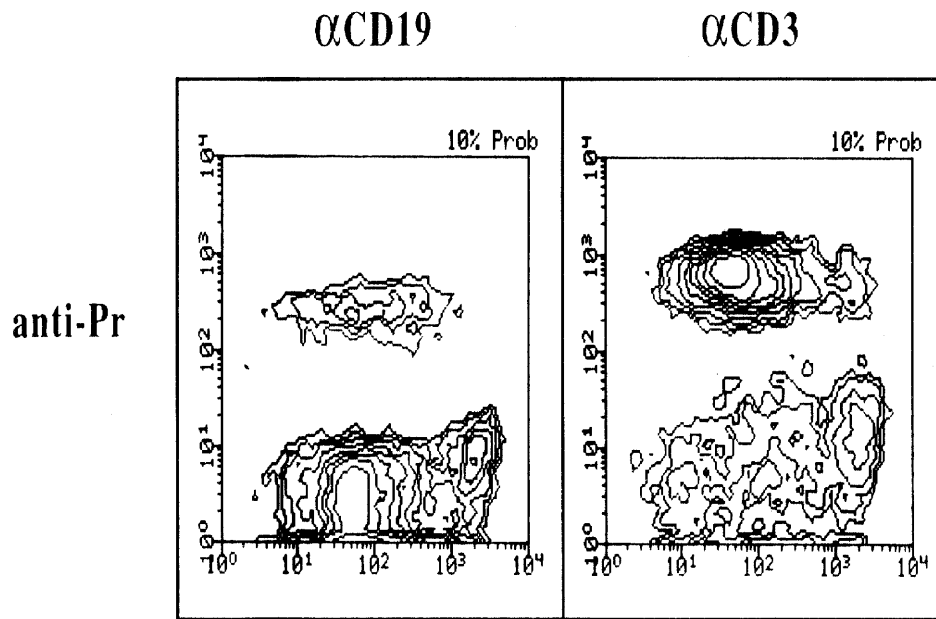


Figure 3. Immunofluorescence analysis of human peripheral blood lymphocytes using two color combinations of reagents specific for Pr₂ (biotinylated anti-Pr₂) and CD19 (PE-conjugated anti-CD19) or CD3 (FITC-conjugated anti-CD3).

Reactivity of Anti-i, I and Pr₂ Autoantibodies to Human Tonsillar Lymphocytes at Various Temperatures

Although autoantibodies are best known for their ability to agglutinate red blood cells at 4°C, we examined whether these antibodies could *bind* lymphocytes also at higher temperatures. Although 15A or LS2 respectively bind 71±4% or 78±4% of tonsillar lymphocytes at 4°C, each

respectively bound 20±2% or 28±12% of lymphocytes at 37°C (p<0.05, one way analysis of variance and Scheffe F-test; Figure 4). Nevertheless, the proportions of lymphocytes bound by these antibodies at 37°C are still significantly higher (p<0.005, Student's t test) than that of control human IgM of irrelevant specificity (which stains less than 0.05 % of lymphocytes).

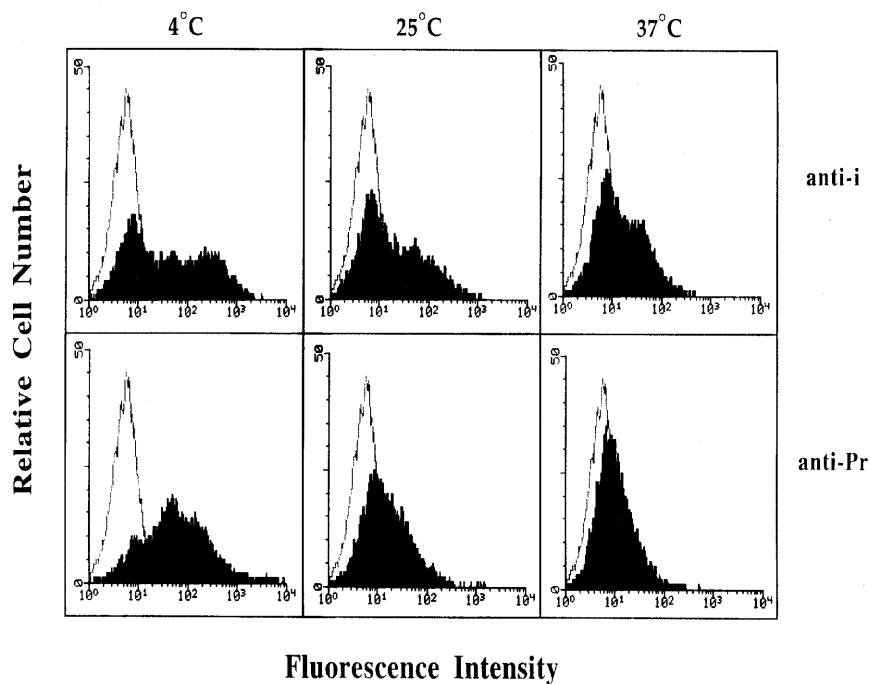


Figure 4. Influence of temperature on anti-i and anti-Pr₂ autoantibody staining of human tonsillar lymphocytes. The top row of histograms (shaded) depicts the logarithmic green fluorescence intensities of lymphocytes stained with anti-i autoantibodies at 4°C, 25°C and 37°C respectively. The bottom row of histograms (shaded) represents anti-Pr staining similarly at 4°C, 25°C and 37°C respectively. The histogram depicted by a solid line represents lymphocytes stained with a control biotinylated human IgM of irrelevant specificity and avidin-FITC.

Binding of Human Anti-i, I and Pr₂ Autoantibodies to Murine Splenocytes and Peritoneal Lymphocytes

Anti-i/I binding. 15A (anti-i) or 20A (anti-I) each displayed the same respective specificity for murine B cells as for human B lymphocytes (Figure 5). 15A stained significantly larger proportions of murine B cells than 20A. For example, data from six staining experiments showed that 15A stained 77±15% of murine splenic B cells, whereas 20A reacted with only 18±13% of such B cells ($p < 0.005$). BALB/c spleen contains very few Ly-1 B cells (21), more recently designated as B-1 B cells (22). To

investigate to what extent these autoantibodies react with the B-1 B cell subpopulation, we also examined peritoneal B cells, as they are known to be enriched for lymphocytes of the B-1 B cell subpopulation. We find that 15A (anti-i) stained 42±14% of the peritoneal B220⁺ lymphocytes, whereas 20A (anti-I) stained only 17±8% of peritoneal B220⁺ lymphocytes (data not shown). Moreover, 15A also binds 63±19% ($n=4$) of murine peritoneal B220⁺ gated cells that co-express Ly-1 (CD5) (figure 6). However, 15A also reacted with non-Ly-1 B cells, as evidenced by its ability to react with most splenic B cells that do not express this surface antigen.

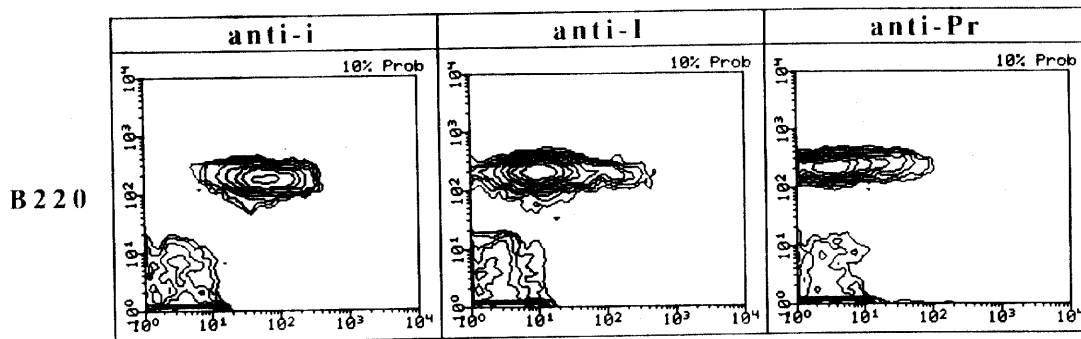


Figure 5. Contour plots depicting the fluorescence of mouse splenocytes stained with PE-conjugated anti-B220 and FITC-conjugated 15A (anti-i), 20A (anti-I), or anti-Pr₂.

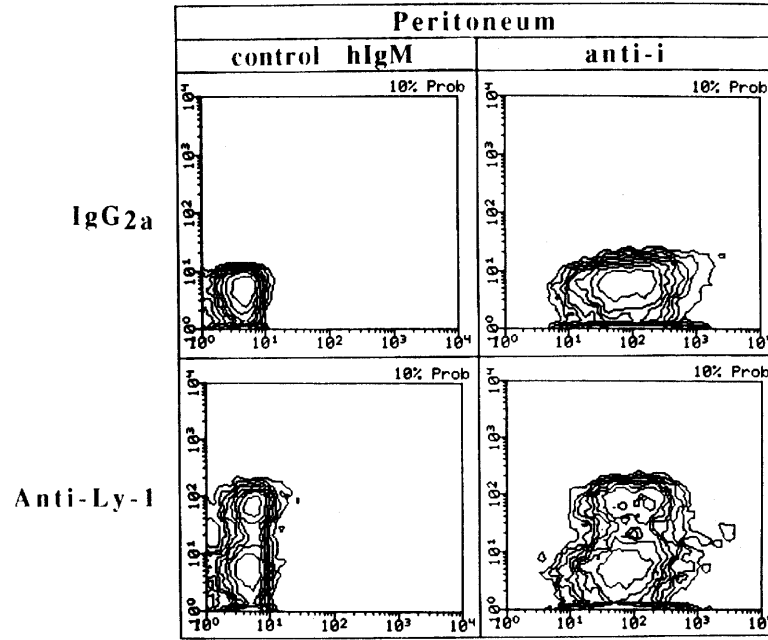


Figure 6. Contour plots of B220+ gated peritoneal B lymphocytes depicting the fluorescence of cells stained with PE-conjugated rat control IgG2a mAb or PE conjugated rat anti-mouse Ly-1 mAb and biotinylated 15A (anti-i) or FITC-conjugated control human IgM.

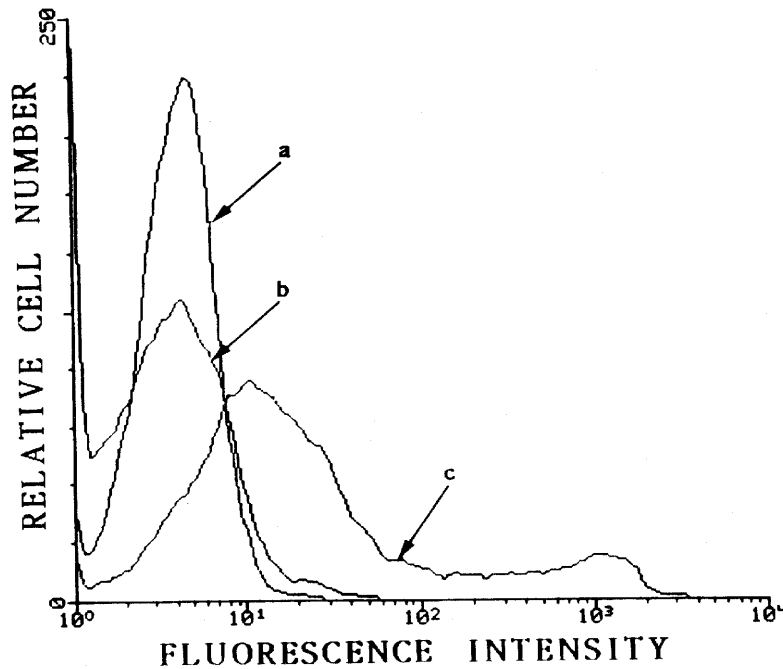


Figure 7. The effect of neuraminidase treatment on anti-Pr₂ staining of tonsillar lymphocytes. Histograms depicting the green fluorescence intensity of CD3+ (PE-conjugated anti-CD3) tonsillar lymphocytes stained with anti-Pr₂-biotin/avidin-FITC. Unstained lymphocytes are depicted by line "a"; untreated (incubated with PBS as control) lymphocytes are depicted by line "c" and neuraminidase treated lymphocytes by line "b".

Anti-Pr₂ binding. The anti-Pr₂ antibody LS2 also reacted with murine lymphocytes (figure 5). In contrast to the staining pattern noted for human lymphocytes, the anti-Pr₂ antibody bound predominantly to murine B cells. Specifically, this antibody reacted with 20-25% of splenic B cells but only 1-2% of the splenic T lymphocytes (data not shown).

Lymphocyte determinants recognized by anti-Pr₂ autoantibodies. We examined whether digestion with neuraminidase affected the determinant on human lymphocytes that is recognized by the anti-Pr₂ antibody. Treatment of red cells with neuraminidase disrupts the red cell α -2,3 linked sialoglycolipids and glycoproteins that are recognized by the anti-Pr₂ antibody (8,23). Similarly, prior treatment of PBL with neuraminidase also disrupts the lymphocyte determinant that is recognized by this antibody (Figure 7). As such, the lymphocyte determinant recognized by the anti-Pr₂ autoantibody also contains sialic acid, indicating that it is similar to the Pr₂ antigen on red blood cells.

Anti-CD45 and Anti-i Antibodies Facilitate the Expression of Secondary Isotypes in T-Dependent Microcultures

To explore the role of the i and I antigens on the B cells in isotype switching, we used a Th cell-dependent B cell stimulation system in which secondary Ig isotype production is efficiently induced by an alloreactive Th cell clone. The majority of isotype switched B cells are consistently found to be derived from cells that were sIgM⁺/sIgD⁺ at the beginning of the culture period and, in many cases, the progeny of single B cells can be observed to switch to multiple isotypes (24,25). Specifically, I-A^b-bearing B cells can be stimulated in clonal microculture by the alloreactive Th cell line, D10, in the presence of DC to secrete a wide range of Ig isotypes. The response is dependent on the addition of all three cellular components: B cells, T cells, and DC.

We distributed 2 cells/well in 60 Terasaki plates and cultured them with 3000 D10 cells and

400 DC in the presence of media alone or media containing various antibodies. Nine days later, the culture supernatants were harvested in 200 μ l RPMI and assayed for secondary isotypes by ELISA. A representative experiment of three is shown in Table 1. The proportion of wells showing the presence of either IgG1 and/or IgA was substantially increased in the presence of anti-B220 or anti-i but not in the presence of anti-I antibody.

Table 1. Anti-B220 as Well as Anti-i Antibodies Induce an Increase in Secondary Ig Isotypes in B-cell Microculture.

B + T + DC ^c	No. Wells Assayed	% Wells Containing IgG or IgA
+ Medium	120	31
+ TIB 146 ^b	120	58 ^e
Anti-i	120	50 ^d
Anti-I	120	39 ^e

^aPeyer's Patch B cells were cultured at two B cells/well with 3000 D10 cells and 400 DC. Cultures were established in 60-well Terasaki plates (Nunc, Intermountain Scientific, Bountiful, CT) and incubated at 5% CO₂ at >95% humidity.

^bAb concentrations added into cultures was 2 μ g/ml. TIB146 is an anti-B220 antibody previously published.

^cp < 0.01

^dp = 0.03

^ep = 0.52

DISCUSSION

We examined autoantibodies specific for i, I or Pr₂ carbohydrate determinants for their ability to bind lymphocytes. Using well-defined, *cell line* derived, human monoclonal antibodies and sensitive multiparameter FACS analysis, we found that anti-i and anti-I antibodies bind only lymphocytes that co-express B cell differentiation antigens. Additionally, the restricted anti-B lymphocyte binding of anti-i and anti-I autoantibodies was observed with both human and mouse lymphocytes. The anti-i antibody bound to a large fraction of mouse splenic B cells and the majority (63 \pm 19%) of CD5⁺ B cells from the peritoneal cavity (figure 6). In contrast, previous studies measuring the cytotoxicity directed by

anti-i and anti-I autoantibodies isolated from serum, suggested that these autoantibodies recognize determinants on both T and B lymphocytes (23,26,27). Conceivably this discrepancy may be due to differences in the source and purity of the antibodies used and/or to differences in the methods employed for detecting the i/I antigen on lymphocytes.

Unlike the B-lineage restricted binding of anti-i and anti-I antibodies, the anti-Pr₂ antibody bound both B and T cells. This was observed for both human and mouse lymphocytes, although compared with human lymphocytes, a much smaller fraction of mouse lymphocytes were bound by this antibody. The Pr₂ antigens, similar to the i/I antigens, are widely distributed on different cell types and are found in many species. However, compared with the i/I antigens, their expression does not appear to change during ontogeny, and little is known regarding their expression on dividing or differentiating cells. It is possible that the Pr₂ antigen may have a physiologic role similar to that of other sialylated oligosaccharides, such as Lewis^x (also initially defined as a red cell antigen), that can serve as ligands on leukocytes for cell adhesion molecules on endothelial cells (28). Sialylated carbohydrate residues (similar to Pr₂) on the CD45RO isoform on T cells also have been shown to bind to the CD22 antigen on B cells, thus facilitating adhesion of B cells to CD4⁺ T cells (29,30). In addition, CD22 mediated adhesion of B cells to other target cells, such as erythrocytes and monocytes, is inhibited by prior treatment of these target cells by neuraminidase (31). Thus, cell membrane bound sialylated oligosaccharides including Pr₂ may be involved in physiologic intercellular interactions, as is the case for CD22.

In this context, the i/I carbohydrate determinants also may be involved in physiologic intercellular interactions. Previously, it was noted that anti-B cell antibodies of patients with Wiskott-Aldrich syndrome could influence B-cell differentiation *in vitro* (32). Such an effect also was observed on murine B cells cultured with antibodies specific for the murine B cell isoform or CD45 (B220) (12). Because the glycosylated

220 kDa B-cell isoform of CD45 possesses the straight chain oligosaccharide of N-acetylglucosamine that forms the i determinant (10,11), and that also constitutes the epitope recognized by V4-34-encoded anti-B cell autoantibodies (1,4) we examined whether anti-i or anti-I also could influence the production of secondary Ig isotypes *in vitro*. We found that addition of anti-i antibody, but not anti-I antibody, to murine splenocytes co-cultured with a stimulator helper T cell clone increased the proportion of B cells secreting secondary Ig isotypes (33,34). Moreover, the effect seen with anti-i was comparable to that observed with anti-B220 mAbs. Taken together, these studies argue that crosslinking the high molecular weight isoform of CD45 on B cells (CD45RA) with anti-i autoantibodies can enhance T-dependent production of secondary Ig isotypes. In this regard, surface Ig with anti-i/B-cell binding activity may play an important role in cognate B-B cell interactions that can facilitate T-dependent B cell maturation/differentiation.

Anti-i expressing B cells (encoded by the V4-34 gene) can be readily isolated from fetal spleen, umbilical cord blood, and adult blood lymphocytes (4,35,36). Furthermore, the V4-34 gene appears over-represented in the expressed Ig gene repertoire of B cells from adult blood (37) or umbilical cord blood (manuscript in preparation). However, the sera of healthy individuals generally contains very low levels of anti-i antibody (38) or antibody reactive with the V4-34 associated cross reactive idiotype, 9G4 (39). Conceivably, B cells expressing V4-34 genes ordinarily do not secrete much soluble Ig, possibly reflecting anergy induced by ubiquitous self antigen and/or absorption of soluble antibody by circulating red blood cells.

Curiously, acute infection with Epstein Barr virus (EBV) is associated with a transient rise of anti-i antibodies, as well as with increased levels of serum Ig bearing the V4-34 associated idiotope 9G4 (40). This suggests that EBV can activate V4-34 expressing B cells, some (or many) of which secrete antibodies with anti-i specificity (3,5,39). Similarly, certain B-cell neoplasms are associated high serum levels of monoclonal anti-i

autoantibodies that can cause hemagglutinin disease (5,41). Such pathologic conditions may reveal an otherwise silent reservoir of B cells that can secrete anti-i autoantibodies. Ordinarily, however, such autoantibodies may be found primarily at the B cell surface where they potentially can play a physiologic role in cognate cell-cell interactions.

ACKNOWLEDGMENTS

Supported by Grants No. DK39065 (L. Silberstein), AG04100 (T. Kipps) and GM48691 (J. Durdik) awarded by the National Institutes of Health.

REFERENCES

1. Grillot-Courvalin C, Brouet JC, Piller F et al. The anti-B cell autoantibodies from Wiskott-Aldrich syndrome recognizes i blood group specificity on B cells. *Eur J Immunol* 22:1781, 1992.
2. Leoni J, Ghiso J, Goni F, Frangione B. The primary structure of the Fab fragment of protein KAU, a monoclonal immunoglobulin M cold agglutinin. *J Biol Chem* 266:2836-2842, 1991.
3. Pascual V, Vistor K, Leisz D et al. Nucleotide sequence analysis of the V regions of two IgM cold agglutinins: evidence that the V_H4-21 gene segment is responsible for the major cross-reactive idiotype. *J Immunol* 146:4385-4391, 1991.
4. Parr TB, Johnson TA, Silberstein LE, Kipps TJ. Anti-B cell autoantibodies encoded by VH4-21 genes in human fetal spleen do not require in vivo somatic selection. *Eur J Immunol* 24:2941-2949, 1995.
5. Silberstein LE, Jefferies LC, Goldman J et al. Variable region gene analysis of pathologic human autoantibodies to the related i and I red blood cell antigens. *Blood* 73:2372-2386, 1991.
6. Hakomori S. Blood group ABH and Ii antigens of human erythrocytes: Chemistry polymorphism, and their developmental change. *Sem Hematol* 18:39-62, 1981.
7. Roelcke D, Ebert W, Giesen H. Anti Pr₃: Serological and immunochemical identification of new anti-Pr subspecificity. *Vox Sang* 30:122-133, 1976.
8. Roelcke D. A Review: cold agglutination. Antibodies and antigens. *Clin Immunol Immunopath* 2:266, 1974.
9. Silberstein LE, Litwin S, Carmack CE. Relationship of variable region genes expressed by a human B-cell lymphoma secreting pathologic anti-Pr₂ erythrocyte autoantibodies. *J Exp Med* 169:1631-1643, 1989.
10. Childs RA, Dalchau R, Scudder P, Hounsell EF, Fabre JW, Feizi T. Evidence for the occurrence of O-glycosidically linked oligosaccharides of poly-N-acetyllactosamine type on the human leucocyte common antigen. *Biochem Biophys Res Commun* 110:424-431, 1983.
11. Childs RA, Feizi T. Differences in carbohydrate moieties of high molecular weight glycoproteins of human lymphocytes of T and B origins revealed by monoclonal autoantibodies with anti-I and anti-i specificities. *Biochem Biophys Res Commun* 102:1158-1164, 1981.
12. George A, Rath S, Shroff KE, Wang M, Durdik J. Ligation of CD45 on B Cells Can Facilitate Production of Secondary Ig Isotypes. *J Immunol* 152:1014-1021, 1994.
13. Silberstein LE, Goldman J, Kant JA, Spitalnik SL. Comparative biochemical and genetic characterization of clonally related human B-cell lines secreting pathogenic anti-Pr₂ cold agglutinins. *Arch Biochem Biophys* 264:244-252, 1988.
14. Judd W, Issitt P, Pavone B, Anderson J, Aminoff D. Antibodies that define NANA-independent antigens. *Transfusion* 12-18, 1979.
15. Fuhrman J, Cebra J. Special features of the priming process for a secretory IgA response: B cell priming with cholera toxin. *J Exp Med* 153:534, 1981.
16. Justement LB, Campbell KS, Chien NC, Cambier JC. Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. *Science* 252:1839, 1991.
17. Kay J, Porcelli J, Tite B, Janeway C, Jr. Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. *J Exp Med* 158:836, 1983.
18. Janeway C, Conrad P, Lerner E, Babisch J, Wettstein P, Murphy D. Monoclonal antibodies specific for Ia glycoproteins raised by immunization with activated T cells. *J Immunol* 132:662, 1984.
19. George A, Cebra JJ. Responses of single germinal-center B cells in T-cell-dependent microculture. *Proc Natl Acad Sci USA* 88:11, 1991.

20. Spradling AC, Mahowald AP. Amplification of genes for chorion proteins during oogenesis in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 77:1096, 1980.
21. Herzenberg L, Stall A, Lalor P et al. The Ly-1 B lineage. *Immunol Rev* 93:81, 1986.
22. Kantor AB. The development and repertoire of B-1 cells (CD5 B cells). *Immunol Today* 12:389-391, 1991.
23. Pruzanski W, Shumak KH. Biological activity of cold-reacting antibodies. Parts I and II. *N Eng J Med* 297:538-542 and 583-539, 1977.
24. Leberman DA, Coffman RL. Interleukin 4 causes iso-type switching to IgE in T cell-stimulated clonal B cell cultures. *J Exp Med* 168:853, 1988.
25. Schrader DE, George A, Kerlin RL, Cebra JJ. Dendritic cells support production of IgA and other non-IgM iso-types in clonal microculture. *Int Immunol* 2:563, 1990.
26. Pruzanski W, Farid N, Keystone E, Armstrong M, Greaves MF. The influence of homogeneous cold agglutinins on human B and T lymphocytes. *Clin Immunol Immunopath* 4:248-257, 1975.
27. Pruzanski W, Roelcke D, Armstrong M, Manly MS. Pr and Gd antigens on human B and T lymphocytes and phagocytes. *Clin Immunol Immunopath* 15:631-641, 1980.
28. Bevilacqua M, Nelson RM. Selectins. *J Clin Invest* 91:379-387, 1993.
29. Stamenkovic I, Sgroi D, Aruffo A, Sy M, Anderson T. The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45RO on T cells and alpha 2-6 sialyltransferase, CD75, on B cells. *Cell* 66:1133, 1991.
30. Munro S, Bast J, Colley K, Tedder T. The B lymphocyte surface antigen CD75 is not an alpha-2,6-sialyltransferase but is a carbohydrate antigen, the production of which requires the enzyme. *Cell* 68:1003-1004, 1992.
31. Engel P, Nijima V, Rothstein D et al. The same epitope on CD22 on B lymphocytes mediates the adhesion of erythrocytes, T and B lymphocytes, neutrophils and monocytes. *J Immunol* 150:4719-4732, 1993.
32. Brouet JC, Grillot-Courvalin C, Seligmann M. Human antibody reacts with a B-cell subset in man to induce B-cell differentiation. *Nature* 283:668-669, 1980.
33. Koretzky G. Role of the CD45 tyrosine phosphatase in signal transduction in the immune system. *FASEB Journal* 7:420-426, 1993.
34. Morikawa K, Oseko F, Morikawa S. The role of CD45 in the activation, proliferation and differentiation of human B lymphocytes. *Int J Hematol* 54:495-504, 1991.
35. Jefferies L, Carchidi C, Silberstein L. Naturally occurring anti-i/I cold agglutinins may be encoded by different VH3 genes as well as the VH4.21 gene segment. *J Clin Invest* 92:2821-2833, 1993.
36. Schutte MEM, van Es JH, Silberstein LE, Logtenberg T. VH4.21-encoded autoantibodies with anti-i specificity mirror those associated with cold haemagglutinin disease. *J Immunol* 151:6569-6576, 1993.
37. Kraj P, Friedman DF, Stevenson F, Silberstein LE. Evidence for the overexpression of the VH4-34 (VH4.21) Ig gene in the normal adult human peripheral blood B cell repertoire. *J Immunol* 154:6406-6420, 1995.
38. Mollison PL, Engelfreit CP, Contreras M. *Blood Transfusion in Clinical Medicine.*, 8th ed. Oxford: Blackwell Scientific Publications, 1988.
39. Stevenson FK, Smith GJ, North J, Hamlin T, Glennie M. Identification of normal B-cell counterparts of neoplastic cells which secrete cold agglutinins of anti-I and anti-i specificity. *Brit J Haematol* 72:9-15, 1989.
40. Chapman CJ, Spellerberg MB, Smith GA, Carter SJ, Hamblin TJ, Stevenson FK. Autoanti-red cell antibodies synthesized by patients with infectious mononucleosis utilize the V_H4-21 gene segment. *J Immunol* 151:1051-1061, 1993.
41. Silberstein LE, Robertson GA, Hannam Harris AC, Moreau L, Besa E, Nowell PC. Etiologic aspects of cold agglutinin disease: Evidence for cytogenetically defined clones of lymphoid cells and the demonstration that an anti-Pr cold autoantibody is derived from a chromosomally aberrant B cell clone. *Blood* 67:1705-1709, 1986.
42. George A, Kerlin RL, Schrader CE, Cebra JJ. *A clonal microculture that supports IgA expression by murine B cells*, Edited by T. T. MacDonald, S. J. Challacombe, P. W. Bland, C. R. Stokes, R. V. Heatley and A. M. Mowat. *Advances in Mucosal Immunology*. London: Kluwer Academic Publishers, 1990.
43. Coffman RL, Weissman IL. A B cell specific member of the T200 glycoprotein family. *Nature* 289:681, 1981.