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Quantitative Expression and Function of Differentiation Antigens on Normal and Malignant Lymphoid Cells

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A. Introduction

As lymphocytes differentiate, the expression of certain cell surface polypeptides can change both quantitatively and qualitatively. A classic example of quantitative variation is murine Thy-1 which decreases over five- to tenfold in density as thymocytes differentiate into mature T cells. What functions these quantitative variations may play for the most part is not known. However, variations in Ia antigen densities can profoundly affect immune responsiveness, and this has been postulated to play a central role in immunoregulation [12]. During lymphoid cell activation, the amount of certain antigens such as CD3 (T3) may decrease dramatically [21], while densities of other antigens may increase [15]. It has been suggested that IL-2 receptors increase in density after T cell activation as a means of promoting autocrine-induced proliferation [11].

A standard method for analyzing normal and malignant lymphoid cell phenotypes is to detect the presence or absence of various cell surface antigens with monoclonal antibodies (MoAb) and immunofluorescent or immunoperoxidase methods. A model put forward by Greaves at the last Wilsede meeting [7] and by others is that lymphoid malignancies suffer "maturational arrest" and that the heterogeneity observed for lymphoid leukemias and lymphomas reflects different stages of normal differentiation. The importance of considering quantitative as well as qualitative differences in leukemia or lymphoma phenotypes has been noted [6, 8], but not uniformly applied. Recently, quantitative flow cytometry and two-color immunofluorescence have been used to phenotype normal lymphocyte populations into more discrete subsets [14, 16, 17]. Here we present a summary of our structural and functional studies of lymphocyte surface structures on normal and malignant B and T cell populations using quantiative two-color flow cytometry.

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B. Materials and Methods

I. Antibodies

The MoAb to B cell-associated antigens used in this study have been described [2, 4, 13]. They include 2H7 and 1F5 specific for the pan-B cell antigen Bp32; 2C3 anti-μ chain; δ-TA4-1 anti-δ chain; HB10a anti-HLA-DR; H616 anti-p76 B cell antigen; 3AC5 anti-p220 pan-leukocyte antigen; and 24.1 anti-p100, CD10 (cALLA) antigen. T cell-specific MoAb described according to the international nomenclature [1, 9] were G19-4 anti-Tp19-29, CD3; G3-7 anti-Tp41, CD7; 9.6 anti-Tp50, CD5 (E receptor); 10.2 anti-Tp67, CD2; G19-2 anti-Tp55, CD4 ($T_{h/i}$); G10-1 anti-Tp32, CD8 ($T_{s/c}$); and 9.3 anti-Tp44. The antibodies were purified and conjugated with fluorescein (green) or phycoerythrin (PE) (red) as described [15, 17].

II. Cell, Preparation, Staining, and Flow Cytometry

Lymphoid cells were purified on Ficoll density gradients and stained with directly conjugated MoAb as described [5, 17]. Because only directly conjugated antibodies of high affinity were used, for cell populations of relatively uniform size, fluorescence intensity is a good indicator of antigen density. For two-color flow cytometry, a FACS IV with a single laser to excite fluorescein or PE was used [15].

C. Results and Discussion

I. Normal B Lymphocyte Populations

The quantitative expression of certain B cell surface polypeptides differs in different lymphoid tissues. For example, both HLA-DR and B4 antigens are expressed at higher densities on splenic and tonsillar B cells than on circulating blood B cells [19, 15]. The pan-B antigen Bp32 is expressed over a range of densities on tonsillar B cells [15]. Using two-color methods, we have found that B cells in tonsils can be classified into three phenotypes: $Bp32^{bright}$ ($Bp32^{bri}$) $IgM^{dull/neg}$ cells, $Bp32^{dull}IgM^{bri}IgD^{-}$ cells, and $Bp32^{dull}IgM^{bri}IgD^{+}$ cells. B cells found in the germinal center of secondary follicles are $Bp32^{bri}IgM^{dull/neg}$ while B cells in the mantle zones are $Bp32^{dull}IgM^{bri}IgD^{+}$ [16]. B cells expressing Tp67 (T1 or Ly1) are of great interest since chronic lymphocytic leukemias (CLL) are $Tp67^{+}$ [18] and $Ly1^{+}$ B cells are elevated in mice with lupus-like autoimmune disease [10]. Using the sensitive two color systems, we have not been able definitively to identify Tp67 B cells in B cell-enriched fractions of cord blood, adult blood, spleen, tonsils, lymph nodes, or bone marrow. If $Tp67^{+}$ B cells are present in normal lymphoid tissues, they are in quite small numbers, are localized in certain areas, or are expressed only at certain times in development.

II. Malignant B Cell Phenotypes

The intensity of antigen expression on chronic lymphocytic leukemias (CLL) and a variety of non-Hodgkin's lymphomas was evaluated [15]. As summarized in Table 1, for CLL antigen, densities for Bp32, sIgM, and HLA-DR vary over roughly a 10- to 25-fold range. The same markers generally are expressed at higher density and vary over a 100- to 500-fold range for the more heterogeneous non-Hodgkin's lymphomas. The Tp67 antigen in contrast is expressed at higher density on CLL than lymphomas. Five of the $Bp32^{+}sIgM^{+}$ samples (16%) expressed significant but low levels ($\geqq 2.0$) of the Tp50 Er marker. In our screen of normal adult lymphoid tissues, we did detect either Er^{+} or $Tp67^{+}$ B cells. Using two-color analyses with Tp67, Bp32, or HLA-DR as markers, at least three distinct phenotypes for CLL were detectable [15].

A small number of non-T, non-B ALL were screened using two-color IF (Figs. 1 and 2). The most informative combination for phemotyping $cALLA^{-}$ ALL was measuring p220 levels versus HLA-DR expression (Fig. 1); three phenotypes were evident: $p220^{bri}DR^{dull/bri}$ (ALL 1); $p220^{bri}DR^{bri}$ (ALL 2); and $p220^{-}DR^{bri}$ (ALL 3). The $cALLA^{+}$ ALL generally had higher levels of HLA-DR and lower levels of p220 than the $cALLA^{-}$ ALL (Table 1). Using green-anti-cALLA versus red-anti-Bp32 or HLA-DR, several phenotypes were detected (Fig. 2). Patient 4's leukemic cells were $cALLA^{dull}Bp32^{dull/neg}$ and $HLA\text{-}DR^{bri}$; patients 5 and 6 both had $cALLA^{bri}Bp32^{dull/neg}$ leukemic cells. However, ALL 5 cells were uniformly DR^{bri} while ALL 6 cells displayed a range of DR densities.

These results show that monoclonal leukemias can be quite heterogeneous in the patterns of surface antigen intensities they display. Apparently, phenotypes indicative of more than one stage of differentiation can be expressed. Two major tasks remain: (a) to relate malignant phenotype to normal lymphoid cell phenotypes – thus far we have not been able to identify the normal cell counterparts of many of our malignant cell phenotypes; and (b) to classify a battery of leukemias and lymphomas based on two-color phenotypes and assess the prognostic value of the classification.

Fig. 1. Quantitative two-color phenotypes of cALLA$^-$ non-T, non-B acute lymphocytic leukemias. Data of 25 000 cells plotted cell number (*vertical axis*) and log green fluorescence (anti-p220) versus log red fluorescence (anti-DR) (*upper left*). About every 4–5 *dots* represents a doubling of fluorescence. ALL 1, a leukemia with p220^{bri}DR$^{dull/bri}$ cells; ALL 2, a second leukemia with p220^{bri}DR^{bri} cells; ALL 3, a third leukemia with p220$^-$DR^{bri} cells

III. Normal T Cells

Two-color cytofluorographic methods have also been used to phenotype new T cell populations. We have conjugated a large panel of MoAb with FITC or PE and tested a variety of two-color combinations. The expression of so-called pan-T cell antigens on CD4$^+$ (T4) T helper/inducer ($T_{h/i}$) cells,

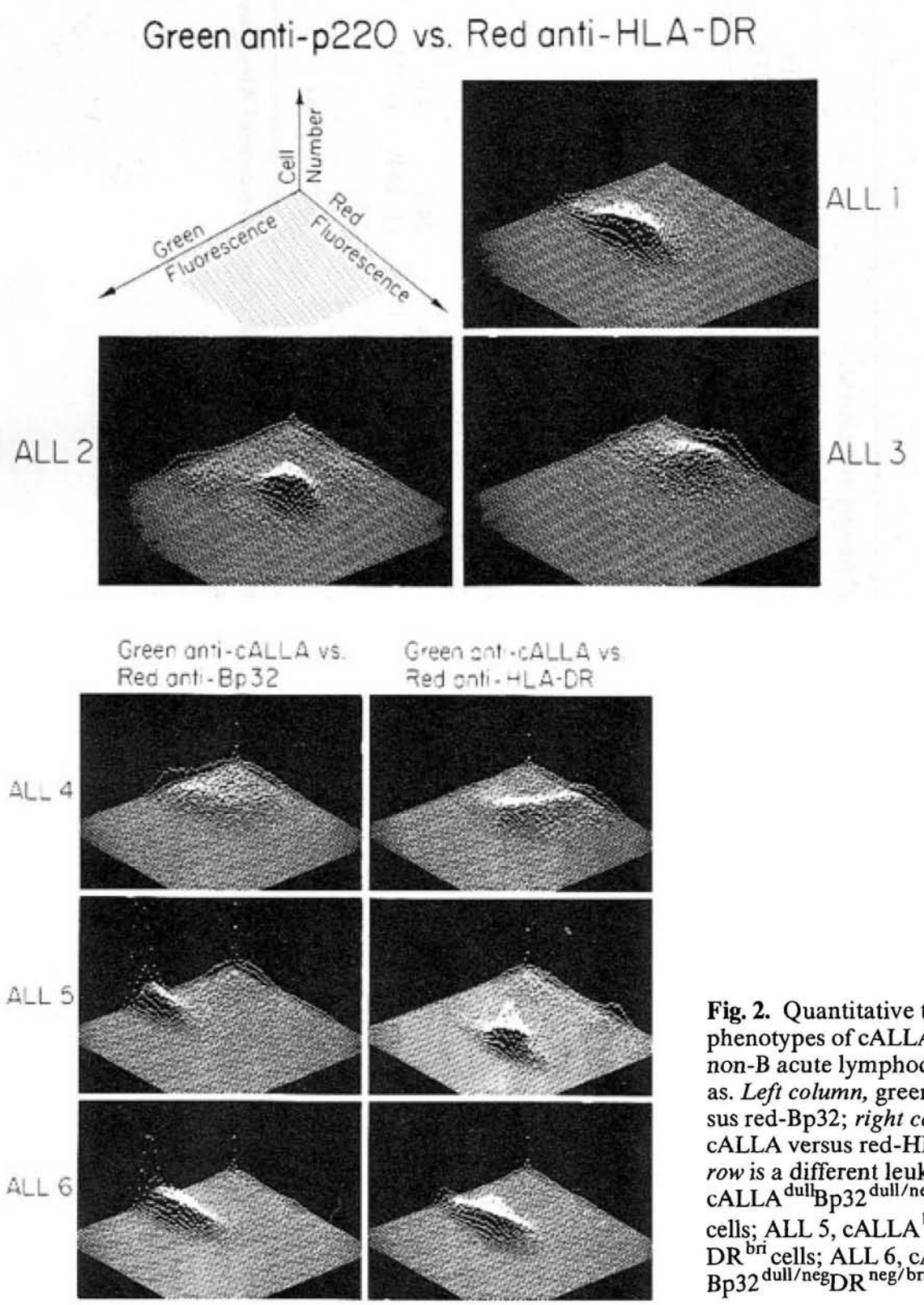

Fig. 2. Quantitative two-color phenotypes of cALLA$^+$ non-T, non-B acute lymphocytic leukemias. *Left column,* green cALLA versus red-Bp32; *right column,* green cALLA versus red-HLA-DR. Each *row* is a different leukemia. ALL 4, cALLA^{dull}Bp32$^{dull/neg}$DR$^{dull/bri}$ cells; ALL 5, cALLA^{bri}Bp32$^{dull/neg}$ DR^{bri} cells; ALL 6, cALLA^{bri} Bp32$^{dull/neg}$DR$^{neg/bri}$ cells

Table 1. Relative antigen expression on B and T cell malignancies

Condition	No.	Mean relative fluorescence intensity[a] (range) using Ab to:										
		Bp32	SIgM	DR	cALLA (CD10)	p220	Tp67 (CD5)	Tp50 (CD2)	Tp19 (CD3)	Tp41 (CD7)	Tp55 (CD4)	Tp32 (CD8)
CLL	15	19.1 (3–49)	1.8 (1–6)	81.3 (8–209)			18.5 (2–42)	2.8 (1–26)				
Non-Hodgkin's lymphomas	16	80.6 (11–189)	65.0 (1–451)	243.3 (19–1154)			5.9 (1–23)	1.5 (1–4)				
ALL												
Non-T, B cALLA$^-$	4	1.0	1.0	19.5 (8–37)	1.4 (1–2)	32.6 (2–63)						
Non-T, B cALLA$^+$	5	2.6 (1–5)	1.6 (1–2)	103.3 (36–215)	54.4 (3–112)	11.5 (3–22)						
T	10	1.1		1.3 (1–2)[b]			8.4 (1–17)	24.1 (1–184)	4.6 (1–22)	98.7 (2–239)	7.3 (1–31)	10.2 (1–56)
Normal PBL-B	3	275.3 (246–314)	15.1 (5–20)	74.5 (54–100)			1.3 (1–2)	1.0				
T		1.4	1.0	1.2			67	47	93	49	51	179

[a] Fluorescence intensity is a ratio of brightness of peak of malignant cells/brightness of peak of negative control Ab-stained cells (autofluorescence). For all experiments, direct IF was used and measured on a FACS IV cell sorter

[b] One Er$^+$ sample, DR = 177

$CD8^+$ (T8) T cytotoxic/suppressor ($T_{s/c}$) cells, and Fc receptor$^+$ (T_γ) T cells on normal periphery was examined [17]. The pan-T cell antigens split into four groups: (a) markers expressed on all $CD4^+$ cells and on $CD8^{bri}$ cells, but not on $CD8^{dull}$ or T_γ cells – CD3 (T3), CD5 (Tp67), and 9.3 (Tp441); (b) markers expressed on all $CD4^+$ and $CD8^+$ cells, but not all T_γ cells – CD2 (Er); (c) markers expressed on all $CD8^+$ and T_γ^+ cells, but not all $CD4^+$ cells – CD7; and (e) markers expressed on all T cell subsets – Tp90. The $T_{h/i}$, $T_{c/s}$, and T_γ cell subsets could be further divided into distinct subpopulations with appropriate two-color combinations (Rose et al., in preparation).

IV. T Cell Malignancies

A panel of ten T cell ALL displayed a range of antigen intensities (Table 1). As reflected by our studies with normal T cells, the most common or widely distributed marker in this panel was the CD7 antigen; CD7 also was consistently expressed at high densities, while many of the other antigens were expressed at lower levels than detected on normal T cells.

V. Functions of Bp32 and Tp32 Polypeptides

The functions of most B and T cell surface antigens are not known; insights into the functions of these molecules may assist in helping us understand leukemogenesis better and may help us design new approaches for diagnosis and therapy. Recently, we have focused on the functions of the B cell-specific polypeptide Bp32 and the $T_{s/c}$-specific polypeptide Tp32, and not solely because we like the number 32 [16]. Monoclonal antibodies to the Bp32 antigen, either alone or in conjunction with T cell factors, stimulate B cells to proliferate. The MoAb act directly on B cells and do not appear to require accessory cells. Anti-μ blocks this effect, suggesting that Bp32 may have to interact with the Ig receptor to activate B cells. Thus, like the CD3 (T3) molecule for T cells, the Bp32 structure plays a role in B cell activation.

The Tp32 molecule is thought to play some role in class I recognition by $T_{c/s}$ cells [20]. Recently, we have demonstrated for the first time that Tp32 molecules physically associate with the class I T cell differentiation antigen Thy,p45 (CD1) on thymocytes [16]. Tp32 appears to be an alternative structure for β_2-microglobulin (β_2M) and class I molecules since β_2M is not associated with the Tp32–Thy,p45 complex. We believe that Tp32 associates with different class I molecules during the course of differentiation and selection/education of cortical thymocytes into mature T cells. At each stage, it is possible that Tp32 plays an important role in allowing or promoting class I recognition.

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