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Correlation of Functional Properties of Human Lymphoid Cell Subsets and Surface Marker Phenotypes Using Multi-parameter Analysis and Flow Cytometry

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INTRODUCTION

Recognition of heterogeneity in lymphocyte populations has been achieved both through the discovery of selective expression of specific cell surface antigens on lymphoid cells and by findings of different functional properties of cells bearing these antigens. The explosive development of hybridoma technology, coupled with the analytical and separation capabilities of fluorescence-activated cell sorting, has led to an extensive classification of human lymphocytes into defined subsets. Of particular importance is the fact that expression of certain cell surface antigens correlates with functional properties or the differentiation state of the cell. Although in most cases the actual physiological role of the cell surface antigen is as yet unknown, monoclonal antibodies can be used to positively or negatively select identified subpopulations of cells for further functional, biochemical or genetic studies. It is anticipated that eventually it should be possible to unequivocally identify only those cells mediating a specific and select function by analysis of their cell surface phenotype.

Several methods have been devised to identify and subsequently separate cells using monoclonal antibodies against cell surface antigens. For negative selection, complement-mediated cytotoxicity assays are used to eliminate subsets of cells in a heterogeneous population. The remaining cells can be

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examined for loss or enrichment of a particular cell function. The disadvantages of this method are that C-mediated cytotoxicity assays often fail to eliminate cells expressing low quantities of cell surface antigen (Ledbetter et al. 1980), and it is often difficult to find sources of complement with low toxicity and high activity. Immuno-adsorption, most commonly used in the "panning" mode (Wysocki & Sato 1978) can also be used for cell separation. In this method, the surface of plastic culture dishes or other solid-phase matrices are coated with antibody so that cells with the appropriate antigen selectively bind to the matrix. This procedure is also best for depletion, but in certain cases can be used for positive selection. Although large numbers of cells can be handled in this procedure, non-specific binding and weak binding of cells expressing low quantities of cell surface antigen are often encountered.

The most certain method of separation involves identification and separation of cells stained with a single or combinations of fluorochrome conjugated antibodies using a fluorescence-activated cell sorter (FACS). The major advantage of this technology is that living cells can be sorted relatively rapidly on the basis of size (using low forward angle and 90 degree light scatter) and specific immunofluorescence (Herzenberg & Herzenberg 1978). It permits cell separation not only on the basis of presence or absence of a particular antigen, but also by the AMOUNT of cell surface antigen. Using multiparameter flow cytometry analysis and sorting, it is possible to directly correlate the function of a cell population with the cell phenotype as defined by multiple immunological and biophysical properties.

In such experiments, however, it is essential to ensure that the actual binding of antibody to the cells does not modify a functional property of the cell under test. With occasional antibodies, such alteration in function can occur by either blocking or stimulation of the receptor, or by modulation of cell-surface antigens. Before describing several specific studies using cell sorting for analyzing specific functional properties, it is perhaps pertinent to briefly review the major functional human T cell subsets.

Studies of two mutually exclusive subsets of human T lymphocytes have contributed significantly to our knowledge of immune regulation (Reinherz & Schlossman 1980). Mature T cells which express either the Leu 3 (T4) antigen or the Leu 2 (T8) antigen comprise the helper/inducer (TH) subset or the suppressor/cytotoxic (TS+TC) subset, respectively. Upon activation with antigen, TH cells provide signals necessary for the differentiation of B lymphocytes into Ig-secreting cells and for the differentiation of Tc and Ts precursors into mature cytotoxic and suppressor cells. Although the correlation between the Leu 2 or 3 phenotype and function has proved useful, it is far from being fully understood, as exemplified by the observation that both Leu 2 and Leu 3 subsets include cells that may become cytotoxic effectors. In fact, the Leu 2/3 phenotype may be primarily associated with the cell's capacity to recognize

particular products of the major histocompatibility complex (MHC). Thus, Leu 3 cells are capable of proliferating in response to MHC Class II allo-antigens (e.g., HLA-DR) and soluble antigens presented in association with Class II determinants (Engleman et al. 1981), and cytotoxic Leu 3 T cells are specific for determinants on Class II molecules (Krensky et al. 1982, Biddison et al. 1982, Meuer et al. 1982). In contrast, Leu 2 T cells do not proliferate in response to most soluble antigens of Class II determinants (Engleman et al. 1981), but can differentiate into cytotoxic cells with specificity for Class I (HLA A,B) molecules (Meuer et al. 1982).

Although cells within each major lineage may share a common MHC receptor, their functional heterogeneity raises the possibility that a given T cell has the potential of mediating several functions, possibly dictated by the nature of the antigenic stimulus with which the cell comes into contact. Alternatively, there may be multiple subsets of mature T cells, each with a unique set of surface markers that correlates with function. In the studies reviewed here, monoclonal antibodies were used to demonstrate the existence of subsets with relatively narrow functional repertoires within the two major T lineages. Although the possibility remains that some T cells are multifunctional, our data favors the concept that most circulating T cells have been programmed to carry out defined tasks which correlate precisely with surface phenotype.

Comparative studies of murine and human cell surface phenotypes, tissue distributions of the different cell types, and biochemistry of the membrane molecules have provided very useful means of determining structure and function of human lymphoid populations (Ledbetter et al. 1981). For example, *in vivo* studies carried out in the mouse, where human studies would be difficult or impossible, can provide valuable information about the homologous cells in the human. Definite establishment of homology between the mouse and human antigens requires amino acid or nucleic acid sequence comparisons. To this end, we (and others) are attempting to clone the genes for some of the T cell differentiation antigens. A successful first step in this direction has been the transfection of mouse fibroblasts with the human Leu 1 and Leu 2 genes using total human DNA (Kavathas & Herzenberg 1983).

A distinct cell function that has also received considerable attention is the natural killer cell activity. In terms of cell lineage of these cells, considerable controversy has developed as to whether these cells are classical T lineage cells, are of non-lymphoid origin, or represent a diverse heterogeneous collection of cells with members in several recognized – and perhaps unrecognized – lineages. Use of monoclonal antibodies and FACS analysis has provided some insight into this problem, but has not yet led to a complete resolution of this matter. In the studies described herein, using monoclonal antibodies, we clearly demonstrate that distinct subpopulations of such cells exist, some of which may show a relationship to the T cell lineage while others may not.

II. REGULATION OF IMMUNOGLOBULIN SYNTHESIS BY A SUPPRESSOR-AMPLIFIER CIRCUIT OF T CELLS

The first indication that the Leu 2 suppressor population can be subdivided came from studies of the regulation of immunoglobulin synthesis induced to autologous mixed leukocyte reactions (AMLR). Although this reaction is traditionally measured in terms of T cell proliferation, it also provides a stimulus for polyclonal IgG and IgM synthesis by autologous B cells. Using a reverse hemolytic plaque assay to measure Ig-secreting cells (plaque forming cells,

TABLE I.
PFC Response Induced by Auto-activated T Cell Populations[a]

Added T Cell Population[b]	Response[c] IgM-PFC	IgG-PFC
No T	14 (1.47)	41 (1.15)
Unseparated T	557 (1.14)	412 (1.1)
Leu 3^{+d}	1067 (1.47)	541 (1.04)
Leu 2^{-d}	1240 (1.14)	666 (1.05)
Leu 2^{+d}	35 (1.61)	48 (1.28)
Leu 3^{-d}	21 (1.49)	48 (1.46)

[a] When unseparated T cells or T cell subsets were cultured along, no PFC were detected.
[b] Microwell cultures consisted of 10^5 non-T stimulators (and B-cell source) and 10^5 of the indicated T cell population. Cells were harvested on day 8 of culture.
[c] Geometric mean of triplicate measurements of plaque-forming cells per 10^6 non-T cells originally placed in culture, with standard deviation in parentheses.
[d] Cells were isolated by positive or negative selection as indicated.

TABLE II.
Suppression of the Leu 2^- 3^+ Induced PFC Response by Leu 2^+ 3^- T Cells

Ratio Leu 2^+ 3^-: Leu 2^- 3^{+a}	% Suppression of the Response[b,c] Mean % (Range)[d] IgM-PFC	IgG-PFC
1:1	42% (37–45)	50% (30–73)
2.5:1	72% (64–79)	66% (49–82)
5:1	74% (60–87)	78% (67–91)

[a] The number of Leu 2^- 3^+ cells was 2×10^5 per microwell.
[b] Percent suppression was calculated by the formula:

$$\frac{\text{Leu } 1^- \; 3^+ \text{ alone} - (\text{Leu } 2^- \; 3^+ + \text{Leu } 2^+ \; 3^-)}{\text{Leu } 2^- \; 3^+ \text{ alone}} \times 100$$

[c] Suppression by Leu 2^+ 3^- cells of the response induced by Leu 2^- 3^+ cells was significant at $p<0.01$ for all ratios shown.
[d] The mean was calculated from experiments using 4 individuals. Range of suppression is indicated in parentheses.

PFC), we showed that induction of Ig secretion has an absolute requirement for Leu 3 cells (Table I), whereas an excess of Leu 2 cells inhibits the response (Table II, Gatenby et al. 1981). Although fresh Leu 2 cells exerted a modest suppressive effect on Ig production, Leu 2 cells that had been primed with AMLR (that is, in the presence of autologous Leu 3 and non-T cells) had a much more potent effect (Gatenby et al. 1982a). As shown in Figure 1, as few as 4,000 primed Leu 2 cells blocked the helper effect of 200,000 T (Leu 2+Leu 3) cells. On the other hand, when the same low number of activated Leu 2 cells were added to cultures containing no fresh Leu 2 cells (Leu 3 cells only), no significant suppression was seen. Moreover, irradiation (1500 rads) of the fresh Leu 2 cells eliminated suppression, but irradiation of only the primed Leu 2 cells had no effect on suppression (Gatenby et al. 1982a). We interpreted these results as indicating that activated Leu 2 cells are not directly inhibitory of fresh cultures, but act to amplify the otherwise weak suppressive effects of fresh Leu 2 cells. Additional results indicated that only those activated Leu 2 cells that expressed HLA-DR (Ia) antigen, an antigen not detectable on resting T cells, but expressed by 5–15% of Leu 2 cells cultured in AMLR, had a suppressor-amplifier effect (Gatenby et al. 1982a).

In summary, as depicted in Figure 2, these experiments produced evidence for Leu 3 T cell-dependent induction of a Leu 2 suppressor-amplifier precursor cell which, when activated, expressed DR antigen. Although Leu 2^+ DR^+ cells

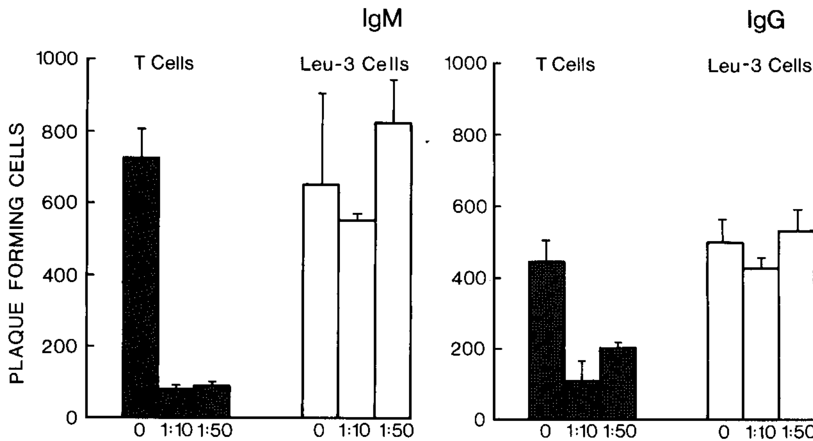

Figure 1. Suppressive effect of activated Leu-2 cells on immunoglobulin production in the autologous MLR: Activated Leu 2 cells from 8-day autologous MLRs were added in varying ratios to fresh cultures containing 10^5 non-T cells and 2×10^5 T or Leu 3 cells per well. The ratios under each column represent the proportion per well of pre-activated Leu 2 cells to fresh T cells or Leu 3 cells. The vertical columns show the mean and standard deviation of the PFC generated per 10^6 non-T cells oroginally placed in fresh microwell culture. IgM-PFC are shown in the left panel and the IgG-PFC in the right.

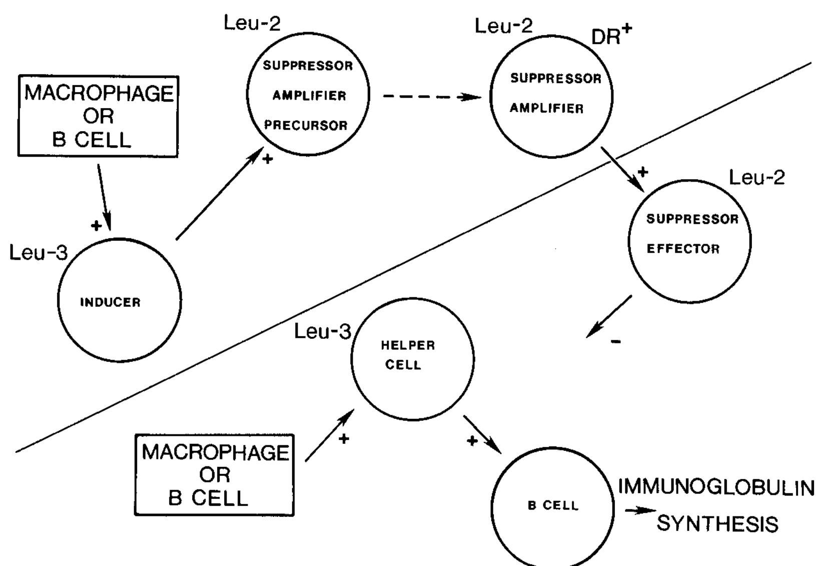

Figure 2. Proposed schema of the immunoregulatory interactions that influence the synthesis of immunoglobulin, in vitro. ——→ + or − indicate positive and negative signals respectively. – –→ indicates a step which may represent the action of one cell upon another, or the maturation of one cell type into another. The surface phenotype defined is marked on each cell type. The events envisaged occurring in the first culture are shown above, those in the second culture below.

exert little or no direct suppression, they can act as potent radioresistant suppressor-amplifiers that greatly enhance the inhibitory effect of fresh Leu 2^+ DR^- cells on Ig synthesis induced in AMLR. This human T cell circuit is analogous in several respects to immunoregulatory networks described previously in the mouse. For example, in the feedback system of Eardley et al., Lyt $1^+2^-3^-$ cells sensitized to sheep erythrocytes activated Lyt $1^+2^+3^+$ cells which, in turn, amplify the inhibitory effects of Lyt $1^-2^+3^+$ (actually Lyt $1(\text{low})2^+3^+$) suppressor-effector cells on the synthesis of anti-erythrocyte antibodies (Eardley 1980). Interestingly, both this system and T cell proliferation induced in AMLR are profoundly impaired in NZB mice, an inbred strain susceptible to a disorder with features similar to systemic lupus erythematosus in man (Eardley 1980, Smith & Pasternak 1978).

III. ROLE OF LEU 8^+ AND LEU 8^- SUBSETS IN THE REGULATION OF Ig SYNTHESIS

Although the above studies indicated the existence of functionally distinct subsets within the suppressor lineage, it was not clear whether these subsets derived from a single resting cell type or from phenotypically distinct resting

cells. In an effort to evaluate the heterogeneity of resting cells within both Leu 2-positive and Leu 3-positive lineages, we began to search for new monoclonal antibodies that recognize some, but not all, Leu 2^+ and/or Leu 3^+ cells. One such antibody, anti-Leu 8, has proven particularly interesting in initial studies (Gatenby et al. 1982b).

Although anti-Leu 8 reacts with most peripheral blood leukocytes including monocytes, granulocytes as well as both B- and T lymphocytes, a small portion of the cells in peripheral blood do not express the antigen, or express the antigen in very low cell surface density. Using this antibody in combination with Leu 2 or Leu 3 it is possible to define several previously undefined subsets of cells. Peripheral blood mononuclear cells were stained with FITC anti-Leu 8, and biotin conjugated anti-Leu 2/Texas Red avidin and then analyzed using a dual laser FACS IV. Lymphocytes were "gated" on the basis of low forward angle and 90 degree light scatter. Within the population of cells reacting with anti-Leu 3, there were cells which reacted strongly with the anti-Leu 8 antibody, and a small subset of cells (7.5% in Figure 3) which reacted weakly or not at all with anti-Leu 8. In general, anti-Leu 8 stains approximately 75–95% of Leu 3^+ cells. Similarly, cells reacting with the anti-Leu 2 antibody also can be subdivided on the basis of Leu 8 reactivity (Figure 3), with 50–80% of the Leu 2-positive cells

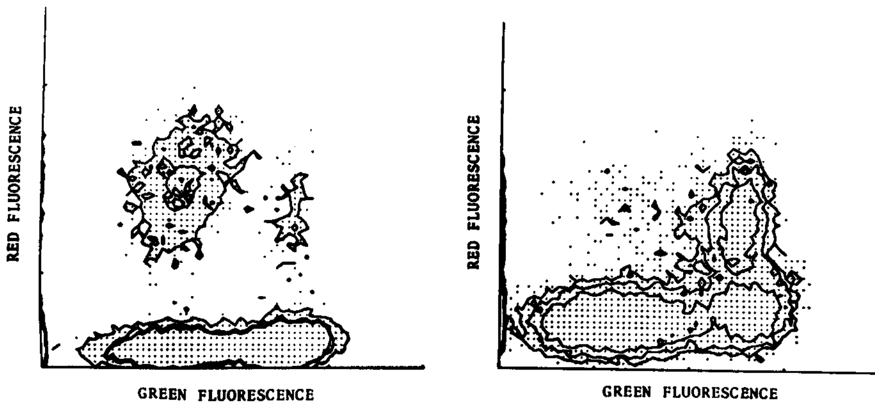

Figure 3. Two color flow-cytometry analysis of human peripheral blood lymphocytes: relationship of Leu 8 expression to Leu 2 and Leu 3. Peripheral blood mononuclear cells were stained with FITC-Leu 8 and biotin-Leu 2a/Texas Red avidin (left panel) or FITC-Leu 8 and biotin-Leu 3a/Texas Red avidin (right panel) and were analyzed by flow cytometry. X axis = log green fluorescence, gain 1; Y axis = linear red fluorescence, gain 0.5 in left panel, gain 1.0 in right panel. Contours were drawn to enclose areas which contained more than 5, 10 and 20 cells. In control contour plots, all cells were in the lower left corner of the plot (not shown). Integration of the 4 subsets shown in the left panel indicated: Leu 8^-, Leu $2a^-$ (30.0%); Leu 8^-, Leu $2a^+$ (24.0%); Leu 8^+, Leu $2a^+$ (9.3%); and Leu 8^+, Leu $2a^-$ (36.7%). Integration of the right panel indicated: Leu 8^-, Leu $3a^-$ (57.6%); Leu 8^-, Leu $3a^+$ (7.5%); Leu 8^+, Leu $3a^+$ (19.9%); and Leu 8^+, Leu $3a^-$ (21.0%).

TABLE III.
Comparison of the helper effect of Leu 3^+ 8^+ and Leu 3^+ 8^- Subsets with unseparated Leu 3^+ cells in the induction of immunoglobulin synthesis in the AMLR.

Helper Cell Source[a]	Experiment 1	Experiment 2
	IgM-PFC	
Leu 3^+	240 (1.2)[b]	197 (1.04)
Leu 3^+ 8^+	157 (1.23)	45 (1.13)
Leu 3^+ 8^-	448 (1.03)	275 (1.08)
Leu 3^+ 8^+: Leu 3^+ 8^- (4:1)	N.D.[c]	195 (1.11)
	IgG-PFC	
Leu 3^+	504 (1.05)	292 (1.09)
Leu 3^+ 8^+	214 (1.18)	127 (1.15)
Leu 3^+ 8^-	1,081 (1.08)	645 (1.01)
Leu 3^+ 8^+: Leu 3^+ 8^- (4:1)	N.D.	319 (1.13)

[a] Each culture contains 10^5 non-T cells and 2×10^5 total putative helper cells, per well.
[b] The numbers represent the geometric mean of triplicate measurements of plaque-forming cells per 10^6 non-T cells originally placed in culture, with standard deviation in parentheses.
[c] N.D.=not done.

reacting strongly with anti-Leu 8. In order to correlate this subdivision of the major subsets with any associated functional properties, T cells were first fractionated into Leu 3 or Leu 2 populations using solid-phase panning, and then further subdivided with anti-Leu 8. To analyze their functional properties, unseparated Leu 3^+ cells, Leu 3^+8^+ or Leu 3^+8^- cells were cultured with autologous non-T-cells for 8 days, after which IgM and IgG PFC were measured. The results of three representative experiments (Table III) show that while both subsets of Leu 3 cells induced immunoglobulin synthesis, the majority of the T cell help for B cell antibody secretion was mediated by the Leu 3^+8^- subpopulation. In addition, when the Leu 3^+8^+ and Leu 3^+8^- cells were admixed in the ratio of 4:1 as occurs in peripheral blood, the number of PFC generated was the same as that generated by unseparated Leu 3^+ cells.

In analogous studies of the suppressor subset, equal numbers of Leu 2^+8^+ or Leu 2^+8^- cells were added to cultures containing Leu 3^+ cells and non-T cells, and IgM and IgG PFC were measured after 8 days. The results of three representative suppression experiments are shown in Table IV and demonstrate that while unseparated Leu 2^+ cells produced a marked decrease in the number of PFC, each subset alone produced little or no suppression. When Leu 2^+8^+ and Leu 2^+8^- cells were admixed in a 1:1 ratio, significant suppression of immunoglobulin secretion was again demonstrated (Table III). Results identical to these have recently been obtained in studies of pokeweed mitogen induced Ig synthesis (G. Kansas & E. Engleman, unpublished observations).

These results demonstrate functionally-heterogeneous subpopulations of resting T cells within both the helper and suppressor lineages and suggest that

individual suppressor T cells act in concert to produce suppression of the immune response rather than undergo a succession of phenotypic changes. It will be important to determine whether the Leu 3^+8^+ subset contains the suppressor-inducer population active in the circuit that controls Ig synthesis in AMLR. It will be equally important to determine if the Leu 2^+8^+ and Leu 2^+8^- cells are precursors of suppressor-amplifier and suppressor-effector cells, respectively, or if these subsets have roles in suppression that are yet to be defined.

IV. MULTIPARAMTER FLOW CYTOMETRY ANALYSIS OF LARGE GRANULAR LYMPHOCYTES WITH MONOCLONAL ANTIBODIES TO HUMAN LEUKOCYTE ANTIGENS

"Natural killer" cells are defined as cells which come from unprimed donors and which are able to lyse certain tumor cell lines *in vitro*. The role of these cells *in vivo* in tumor surveillance and homeostatic immune regulation is a matter of considerable interest and controversy (Herberman 1980). Multiparameter analysis has also provided insight into the phenotype and function of cells expressing "natural killer" activity. Using the anti-Leu 7 monoclonal antibody, single parameter analysis shows that about 5–30% of human peripheral blood lymphocytes express the Leu 7 (HNK-1) antigen (Abo & Balch 1981). In the light of other cell-sorting studies on NK function using light scatter paramters

TABLE IV.

Collaboration of Leu 2^+ 8^+ and Leu 2^+ 8^- cells to produce optimum suppression of immunoglobulin synthesis in the AMLR.

	Experiment 1	Experiment 2
	IgM-PFC	
Control [a,b]	1,358 (1.03)	149 (1.13)
Leu 2^+	410 (1.08)	21 (1.53)
Leu 2^+ 8^+	1,028 (1.14)	137 (1.12)
Leu 2^+ 8^-	986 (1.10)	139 (1.26)
Leu 2^+ 8^+: Leu 2^+ 8^- (1:1)	428 (1.13)	23 (1.26)
	IgG-PFC	
Control	1,377 (1.04)	1,024 (1.21)
Leu 2^+	400 (1.08)	243 (1.12)
Leu 2^+ 8^+	1,101 (1.03)	795 (1.11)
Leu 2^+ 8^-	923 (1.04)	780 (1.12)
Leu 2^+ 8^+: Leu 2^+ 8^- (1:1)	317 (1.41)	240 (1.24)

[a] Each culture contained 10^5 non-T cells per well, 2×10^5 Leu 2^- 3^+ cells and 4×10^5 of the putative suppressor population.

[b] Control cultures contained 4×10^5 Leu 2^+ 3^- cells irradiated to 2000 rads, a dose which we have shown to completely ablate any suppressive effect.

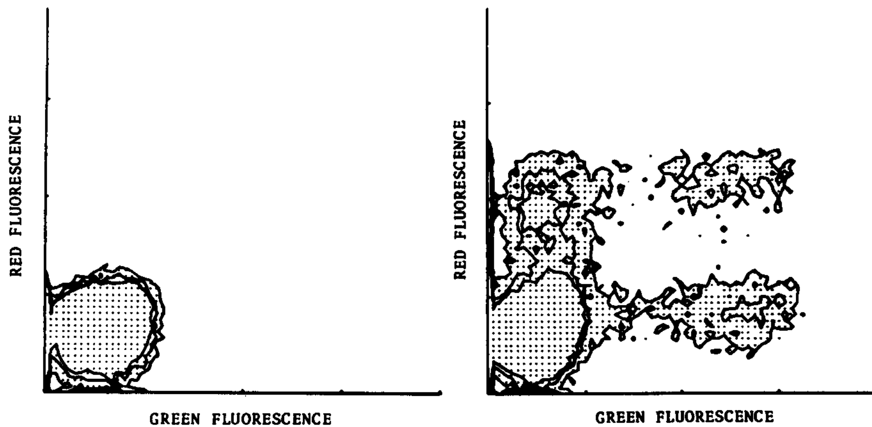

Figure 4. Two color flow-cytometry analysis of human peripheral blood lymphocytes: relationship of Leu 7 and Leu 2a. Peripheral blood mononuclear cells were stained with the FITC-Leu 7 and biotin-Leu 2a/Texas Red avidin (right panel). Control cells (left panel) were stained with a FITC-isotype matched control antibody and Texas Red-avidin. Analysis was performed by flow cytometry. X axis = log green fluorescence, gain 1; Y axis = log red fluorescence, gain 0.9. Contours were drawn to enclose areas which contained more than 5, 10, and 20 cells. Integration of the 4 subsets indicated the following: Leu 7^-, Leu $2a^+$ (17.1%); Leu 7^-, Leu $2a^-$ (62.2%); Leu 7^+, Leu $2a^-$ (11.6%); and Leu 7^+, Leu $2a^+$ (9.1%).

(Warner & Tai 1981), it seemed unlikely that all cells reacting with this antibody were functionally active NK cells. Furthermore, it has been shown that at least some of the cells in peripheral blood lacking the HNK-1 antigen can express a significant proportion of NK function (Lanier et al. 1983a). There are some cells expressing the HNK-1 antigen that also overlap with cells expressing pan T cells markers (e.g. Leu 1 and Leu 4) (Abo et al. 1982, Lanier et al. 1983a). In order to directly examine this situation, two color immunofluorescent staining and FACS analyses have been performed. As shown in Figure 4, a major subset of Leu 7-positive lymphocytes also reacted with anti-Leu 2a monoclonal (about 40% of Leu 7^+ cells co-expressed Leu 2a in most individuals). Also, the Leu 7-positive cells which reacted with anti-Leu 2a expressed a high cell surface density of the Leu 2a antigen. These Leu 7^+ $2a^+$ cells also co-expressed Leu 1 and Leu 4 (Lanier et al. submitted). In contrast, only a very minor subset of Leu 3a-positive lymphocytes expressed the HNK-1 (Leu 7) antigen (about 0.5% of the total lymphocytes in most individuals).

Recently, a monoclonal antibody (NKP-15), designated anti-Leu 11a, generated against Percoll-gradient-purified large granular lymphocytes (LGLs), has been shown to recognize essentially all functional natural killer cells in human peripheral blood (Phillips & Babcock 1983, Lanier et al. 1983b). This antibody reacts with some 5–15% of peripheral blood lymphocytes, all Percoll-

gradient-purified LGLs, and neutrophils, but not resting T cells, B cells, monocytes, eosinophils, mitogen-activated T cells or erythrocytes. Additionally, this antibody prevents the binding of heat-aggregated immunoglobulin complexes to the cell surface of granulocytes and LGLs and may actually recognize the Fc receptor expressed by these cells (Phillips & Babcock 1983). A direct examination of the relationship between the cells reacting with the anti-Leu 7 (HNK-1) and anti-Leu 11a antibodies indicates the existence of four discrete subpopulations of lymphocytes (Leu 7^- $11a^-$; Leu 7^- $11a^+$; Leu 7^+ $11a^+$; and Leu 7^+ $11a^-$) (Figure 5). No Leu 11a-positive cells co-expressing *high* cell-surface density Leu 2a have been observed, although cells co-expressing Leu 11a and *low* cell surface density of the Leu 2a antigen have been demonstrated (1.5% of lymphocytes are Leu $11a^+$, low Leu $2a^+$ in Figure 5). Indirect evidence suggested that the population of cells with the Leu 7^+, Leu $11a^-$ phenotype were mainly comprised of cells expressing high density Leu 2a antigen (e.g. Leu high $2a^+$ 7^+ $11a^-$). There was no significant overlap between cells expressing Leu 11a and those expressing Leu 1, Leu 4, Leu 3, surface immunoglobulin, DR or DC (Leu 10) antigen, although minor overlapping populations (<2% of peripheral-blood lymphocytes) may exist (Lanier et al. 1983a). Hence, these observations permit separation of populations of lymphocytes expressing NK-associated

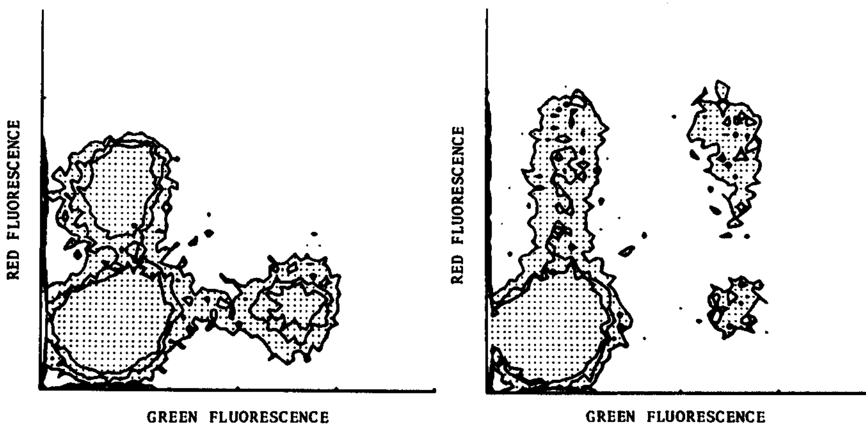

Figure 5. Two color flow-cymtometry analysis of human peripheral blood lymphocytes: relationship of Leu 11a expression to Leu 2 and Leu 7. Peripheral blood mononuclear cells were stained with FITC-Leu 11a and biotin-Leu 2a/Texas Red avidin (left panel) or FITC-Leu 11a and biotin-Leu 7/Texas Red avidin (right panel) and were analyzed by flow cytometry. X axis=log green fluorescence, gain 1; Y axis=log red fluorescence, gain 0.9. Contours were drawn to enclose areas which contained more than 5, 10 and 20 cells. Control contour plot shown in Figure 3. Integration of the 4 subsets shown in the right panel indicated the following: Leu $11a^+$, Leu 7^- (6.1%); Leu $11a^+$, Leu 7^+ (9.8%); Leu $11a^-$, Leu 7^+ (12.9%); and Leu 11a, Leu 7^- (71.2%). Integration of the subsets in the left panel indicate: Leu $11a^+$, Leu $2a^-$ (13.2%); Leu $11a^+$, Leu $2a^+$ (1.5%); Leu $11a^-$, Leu $2a^+$ (24.3%); and Leu $11a^-$, Leu $2a^-$ (61.0%).

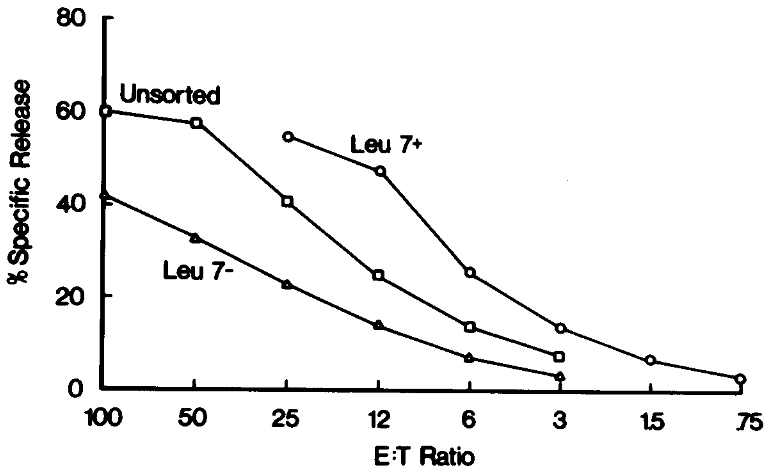

Figure 6. NK activity of Leu 7⁺ and Leu 7⁻ lymphocytes. Peripheral blood mononuclear cells were stained with FITC-Leu 7 and were sorted into negative and positive subsets using a FACS IV cell sorter. Re-analysis of the sorted populations revealed >95% purity. These cells were washed and placed into a 4 hour ^{51}Cr release NK assay using K562 as the target cell line. The results were expressed as % specific ^{51}Cr release (y axis) versus effector to target ratio. (Average maximum release 17,000 cpm; average background release 1,200 cpm).

antigens for further functional studies, and also permit quantitation of these subsets in various disease states associated with suppressed or enhanced natural killer function.

With regard to phenotypic analysis of human lymphocytes, it is important to note that considerable variation between various individuals and in one individual over a period of time has been observed in antigen density and percent of positive cells. This variation is not unexpected, given the genetic heterogeneity and variety of environmental factors known to influence the dynamic state of leukocyte populations and the immune response. These studies clearly demonstrate that multiparameter FACS analysis of lymphocyte subsets further expands the apparent cellular heterogeneity in a manner similar to immunochemical analysis using 2-dimensional rather than single dimension gel electrophoresis. We are studying further the normal percentages of these multiple subsets in order to provide bases for correlation with various disease states.

V. FUNCTIONAL STUDIES OF HUMAN SUBPOPULATIONS EXPRESSING NK-ASSOCIATED ANTIGENS

Using the monoclonal antibodies described above, we have examined the heterogeneity of cell populations capable of mediating NK function. In preliminary studies using the anti-Leu 7 and Leu 11a antibodies, it was evident

that both antibodies could be used to identify populations of cells with NK function. In experiments using the anti-Leu 7 antibody, peripheral blood cells were stained with FITC anti-Leu 7 and then sorted into Leu 7$^-$ and Leu 7$^+$ subsets using a FACS IV cell sorter. Re-analysis of the sorted populations indicated that they were >95% pure. The Leu 7-positve population was considerably enriched for NK activity, relative to the unsorted cells (Figure 7). However, it was also evident that a significant proportion of NK activity was still present in the Leu 7-negative population (approximately 15–20% of NK activity). Similar studies using anti-Leu 7 and rabbit complement for cytotoxic depletion of NK cells have provided identical results, i.e., some residual NK function remains in the Leu 7-negative population. Controls indicated that the binding of anti-Leu 7 monoclonal antibody to the Leu 7-positive NK cells or presence of the anti-Leu 7 antibody in the assay medium did not influence the function of these cells.

In contrast, experiments using anti-Leu 11a antibody demonstrated that essentially *all* NK function is within the population of cells expressing the antigen defined by this monoclonal antibody. We found that peripheral blood mononuclear cells stained with FITC anti-Leu 11a and sorted into positive and negative fractions using a FACS IV cell sorter yielded some 15% of the

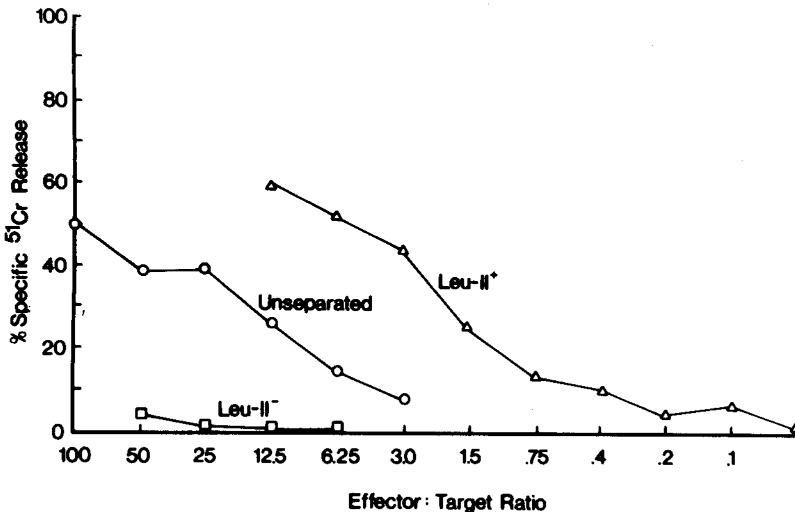

Figure 7. NK activity of lymphocyte subsets defined by two color FACS cell sorting using Leu 11a and Leu 7. Peripheral blood mononuclear cells were stained with FITC-Leu 11a and were sorted into negative and positive subsets using a FACS IV cell sorter. Re-analysis of the sorted populations revealed >95% purity. These cells were washed, and placed into a 4 h ^{51}Cr release NK assay using K562 as the target cell line. The results were expressed as % specific ^{51}Cr release (y axis) versus effector to target ratio (x axis). (Average maximum release 21,249 cpm; average background release 2,221 cpm).

lymphocytes in the positive-sorted fraction. Nevertheless, we found virtually all of the NK function in this Leu 11a-positive fraction (Figure 7). Morphological analysis of this fraction showed that it consisted predominately of large granular lymphocytes. The Leu 11a-negative cells, which comprised a majority of the total lymphocytes, had no NK activity even at an E:T ratio of 50:1 (Figure 7). This finding is highly reproducible with different donors using both FACS and panning cell-separation procedures (Phillips & Babcock 1983, Lanier et al. 1983b). Additionally, we found that all cells capable of mediating antibody-dependent cellular cytotoxicity (ADCC) were contained within the Leu 11a subset (Lanier et al. 1983a).

To further define the phenotype of NK cells, two color sorting was performed using FITC anti-Leu 11a and biotinylated anti-Leu 7 with Texas Red avidin to separate the four subpopulations. All sorted populations were >90% pure when re-analyzed. Cells from the four subsets were assayed for NK function using ^{51}Cr labelled K562 cells as targets. The Leu 7^- $11a^-$ cells were totally incapable of mediating target lysis (not shown). In contrast, the other three populations had at least some NK activity (Figure 9). On a per cell basis, the most efficient functional cells were Leu 7^- $11a^+$. These cells account for the NK activity seen previously in the Leu 7-negative subpopulation. Although these cells comprise a relatively small proportion of the mononuclear cells in peripheral blood, they are very efficient in NK-mediated lysis. In contrast, the Leu 7^+ $11a^-$ and Leu 7^+ $11a^+$ cells were less efficient mediators of NK function than the Leu 7^- $11a^+$ cells.

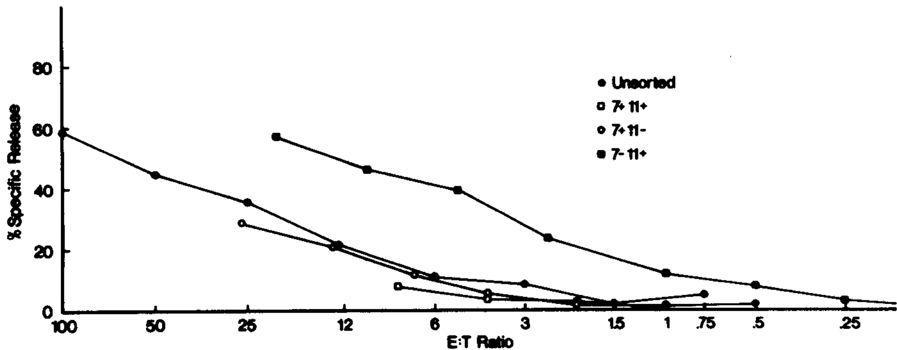

Figure 8. NK activity of lymphocyte subsets defined by two color facs cell sorting using Leu 11a and Leu 7. Peripheral blood mononuclear cells were stained with FITC-Leu 11a and biotin-Leu 7/Texas Red avidin. The 4 subsets shown in Figure 4 were separated by two color cell sorting using a dual laser FACS IV. After sorting the purity of the resulting subpopulations was >90%, as determined by re-analysis using the FACS IV. These cells were washed, and placed into a 4 hour ^{51}Cr release NK assay using K562 as the target cell line. The results were expressed as % specific ^{51}Cr release (y axis) versus effector to target ratio (x axis). (Average maximum release 21,249 cpm; average background release 2,221 cpm).

This latter finding was quite surprising, since in a single stain anti-Leu 7 sort, significant NK activity was found in the Leu 7-positive population. Yet in the two color sort, neither the Leu 7^+ $11a^+$ nor the Leu 7^+ $11a^-$ subsets demonstrated potent cytotoxic potential. There are at least three possible explanations for this data:

1) The binding of the anti-Leu 11a antibody to the Leu 7^+ 11^+ cells may inhibit the function of these cells.
2) The Leu 7^+ $11a^+$ and Leu 7^+ $11a^-$ populations act in concert to effect NK activity.
3) In some individuals, the Leu 7^+ $11a^+$ cells are non-functional.

The first hypothesis was experimentally tested in the following manner. Peripheral blood cells were stained with FITC-anti-Leu 7 antibody and sorted into Leu 7^- and Leu 7^+ subsets. The Leu 7^+ cells were assayed directly for NK activity or were first stained with anti-Leu 11a antibody and then assayed at various E:T ratios. Additionally, anti-Leu 11a antibody was placed into cell-culture medium in the NK assay of the Leu 7^+ subset to determine the influence of the continual presence of this antibody on cell activity. The NK activity of the Leu 7^+ or Leu 7^- subsets was *not* inhibited by the anti-Leu 11a antibody, compared to an isotype-matched irrelevant monoclonal antibody control. Studies to test the second and third hypotheses are presently underway. It is possible that synergistic relationships and previously unrecognized immunoregulatory interactions may be uncovered.

VI. CONCLUSIONS

The studies presented in this article demonstrate some new examples of functionally and phenotypically different human lymphoid-cell populations. In both of the functional systems presented, the T cell helper-suppressor-inducer system and the natural killer system, the use of multiple monoclonal antibodies with multiparameter FACS analysis and sorting led to the identification of complexity within the previously recognized subsets. In both instances, we found a distinct correlation of specific functional properties with the membrane phenotypes of the cells.

Although the T cell population as a whole carriers out diverse regulatory and effector functions, our data shows clearly that individual T cells have limited functional repertoires. The fact that each functional program is associated with a unique combination of surface antigens raises the possibility that the antigens play direct roles in T cell activation/function. In this regard, the observations that antibodies to the Leu 2 and Leu 3 molecules inhibit the MHC-restricted functions of their respective subsets led us (Engleman et al. 1981, 1983, Evans et al. 1981) and subsequently others (Krensky et al. 1982, Biddison et al. 1982,

Meuer et al. 1982) to suggest that these markers may serve as ligands for Class I and II MHC determinants, respectively. Other markers appear to be associated with a T cell receptor for nominal antigens (Meuer et al. 1983), with T cytotoxic potential (Krensky et al. 1983), and with a receptor for T cell growth factor (Leonard et al. 1982). The roles of most T cell markers, including Leu 8 and 9.3, have yet to be determined.

In the studies with natural killer cells using multiparameter FACS analysis and sorting, we directly demonstrated the existence of several subsets of lymphocytes expressing "NK-associated" cell surface antigens, including: Leu 7^- 11^+; Leu 7^+ 11^+; Leu 2^- 11^+; Leu $2a^+$ 11^+ (low density Leu 2a); Leu 2^+ 7^+ (high density Leu 2a); and Leu 7^+ 11^-. Preliminary studies suggest that the Leu $2a^+$ 7^+ $11a^-$ subset also expresses the Leu 1 and Leu 4 antigens, thus implying that these cells are actually derived from the T lymphocyte lineage. The functional activities and inter-relationships of these subsets are of considerable interest both theoretically and practically. In certain individuals, the Leu 7^+ 11^+ and Leu 7^+ 11^- subsets alone express very little NK activity. The data presented herein indicates that on a per cell basis, the most efficient mediators of NK activity are the Leu $11a^+$ 7^- cells. However, in certain individuals, this population can be further subdivided on the basis of low-density expression of Leu 2a antigen. There are also indications that the Leu $2a^+$ 7^+ (high Leu 2a antigen density) population may represent a unique subset of T lymphocytes, which interact with functional NK cells. Thus, using such a multifaceted approach, it may be possible to uncover the mechanism of NK activity and to understand the regulation and effector functions involved in the NK system.

With respect to the possible clinical applications of these results, it is apparent that immunoregulation and effector functions in man result from a complex series of cellular interactions. Consequently, simple determination of the frequency of major subsets such as Leu 2 and Leu 3 in peripheral blood, or measurement of a defective proliferative response to antigens or mitogens will probably not be adequate for understanding immunological-mediated disorders. As noted earlier, both the proliferative response in autologous MLR and the feedback suppressor circuit of immunoglobulin synthesis are defective in the NZB mouse model. Although these animals maintain a normal number of Lyt 1^-low 2^+3^+ cells, they lack the Lyt $1^+2^+3^+$ suppressor-amplifier cell. It will be of interest to determine whether this cell type is present and functionally intact in patients with diseases characterized by disordered immunity. However, with the availability of antibodies such as anti-Leu 7, Leu 2a, Leu 3a, Leu 8, and Leu 11a, which subdivide the leukocytes into multiple subsets, it should be possible to more critically determine the frequency and distribution of the specific cell types in immunologically-mediated diseases and to assess their roles *in vivo* in health and disease.

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