Lymphocytes in Patients with Variable Immunodeficiency and Panhypogammaglobulinemia

EVALUATION OF B AND T CELL SURFACE MARKERS AND A PROPOSED CLASSIFICATION

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ABSTRACT Peripheral blood lymphocytes from 15 patients with variable immunodeficiency and severe panhypogammaglobulinemia were evaluated for B and T cell surface markers. B cells were enumerated by immunofluorescent detection of both surface immunoglobulin (Ig) and the ability to bind aggregated Ig complexes. T cells were identified by their ability to form nonimmune rosettes with sheep red blood cells. Four distinct patterns were observed which were designated types I–IV. Type I: six patients had normal percentages (8.5–19.0%) of Ig-bearing B lymphocytes. Type II: four patients were observed to have B lymphocytes (4.5–15.0%) which lacked fluorescence-detectable surface Ig. Type III: the peripheral blood of these four patients contained a subpopulation (11.3–20.0%) of lymphocytes which apparently lacked both B and T cell markers ("null" cells). Type IV: one patient's blood was characterized by a subpopulation (18.0–22.0%) of lymphocytes which bore both B and T cell markers. Patients of each type had some clinical features in common. It is concluded that evaluation of lymphocyte surface markers provides a means of separating patients with variable immunodeficiency and panhypogammaglobulinemia into distinct groups which appear to differ in the nature of their fundamental defect.

INTRODUCTION

The majority of patients with primary immunodeficiency remain unclassified and are referred to as "variable immunodeficiency" (1). Despite detailed clinical studies (2), and extensive studies of serum immunoglobulin (3), little is known of the basic defect(s) involved. Thus, it was apparent that further advances required studies at a cellular level.

Lymphocytes are composed of two main populations: thymus-derived (T)[1] lymphocytes which are largely responsible for cell-mediated immunity, and bursal-equivalent or bone marrow-derived (B) lymphocytes which are primarily involved in humoral immunity. Identification of these populations is a necessary first step in cellular studies of immune deficiencies. B cells can be identified by the presence of easily detectable surface immunoglobulin (Ig) (4–6), and by their ability to bind antigen-antibody complexes or heat-aggregated Ig via the Fc portion of the Ig molecule (7–10). Human T cells can be identified by their ability to form nonimmune rosettes (E rosettes) with sheep red blood cells (SRBC) (11–14). Several laboratories have reported that enumeration of Ig-bearing lymphocytes in patients with variable immunodeficiency has led to the delineation of at least two different patterns (normal to near normal, or few to absent) presumably reflecting different underlying defects (15–18).

The present communication reports evaluation of the peripheral blood lymphocytes (PBL) from 15 patients with variable immunodeficiency and severe panhypogammaglobulinemia for surface Ig, aggregated Ig binding, and E rosette formation. Several distinct patterns (types) were observed each of which suggested a different underlying defect. Furthermore, patients of each type

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[1] *Abbreviations used in this paper:* B, bursal equivalent; E rosettes, nonimmune rosettes; Ig, immunoglobulin; PBL, peripheral blood lymphocytes; SRBC, sheep red blood cells; T, thymus derived.

had some clinical features in common. These data are used as the basis of a proposed classification of variable immunodeficiency with panhypogammaglobulinemia.

METHODS

Patients. 15 patients were selected who were severely panhypogammaglobulinemic and met the criteria of "variable immunodeficiency" according to the WHO recommendation (1). No history of X-linkage could be confirmed in any of the cases.[2] Each patient was studied only when free of acute infection. Most patients were studied on more than one occasion (weeks to months apart) and no changes in the results were observed over time. Serum immunoglobulins were evaluated and quantitated using immunoelectrophoresis and single radial immunodiffusion (19). Cell-mediated immunity was evaluated by skin tests to mumps, PPD (purified protein derivative), Candida, histoplasmin, and in certain cases, dinitrochlorobenzene sensitization. In most cases, T cell function was also evaluated by [^{3}H]thymidine incorporation of lymphocytes stimulated by phytohemagglutinin (kindly performed by Dr. David L. Nelson).

Lymphocyte surface markers. Lymphocyte separation and fluorescent detection of surface Ig and aggregated Ig binding were performed as previously described (8, 9). Briefly, PBL were isolated from venous blood by Ficoll-Hypaque density flotation followed by passage over nylon fiber columns. The purified PBL were then incubated with polystyrene beads and cells adhering or phagocytizing beads were subsequently excluded from evaluation of B and T cell markers. After thorough washing, the PBL were incubated with either rhodamine-conjugated anti-human Ig (specific for γ, μ, α, κ, λ) or fluorescein-conjugated, heat-aggregated human immunoglobulin, washed again thoroughly, and evaluated by alternate phase contrast and fluorescence microscopy for the percentage of positive cells.

E rosettes were prepared by a previously described modification (20) of the technique of Weiner, Bianco, and Nussenzweig (14). Purified PBL and neuraminidase treated SRBC were incubated together at 37°C, pelleted at 200 *g*, and incubated at 4°C for a minimum of 30 min. The pellet was gently resuspended and counted by phase con-

[2] The clinical presentation in two patients (Roz, Bad) resembled that of the infantile, X-linked type but with negative family history.

TABLE I

B and T Lymphocyte Surface Markers on Peripheral Blood Lymphocytes from Patients with Variable Immunodeficiency and Panhypogammaglobulinemia

Patient	Surface Ig	Aggregated Ig binding	E rosettes	Totals*
Normal controls‡				
Mean	20.2§	19.2	77.5	96.7–97.7
Range	6.3–39.5	6.8–38.0	55.0–91.0	89.0–102.5
Type I				
Per	19.0	18.5	76.0	94.5–95.0
Min	18.0	17.5	76.5	94.0–94.5
Sch	11.5	9.5	88.0	97.5–99.5
Phi	10.0	9.0	89.0	98.0–99.0
Roz	9.5	9.0	87.5	96.5–97.0
Wal	8.5	9.0	88.0	96.5–97.0
Type II				
Sco	0.5	15.0	86.0	101.0
Mck	1.0	10.0	84.0	94.0
Pas	0.25	9.5	81.0	90.5
Cat	1.0	4.5	90.0	94.5
Type III				
Ste	6.0	5.0	75.0	80.0–81.0
Wag	7.0	7.5	75.2	82.2–82.7
Kil	5.5	5.0	82.0	87.0–87.5
Bad	10.7	9.5	78.0	87.5–88.7
Type IV				
And	36.7	34.0	80.5	114.5–117.2

* Totals obtained by addition of percentages for B and T cell markers. In type II only aggregated Ig binding percentages were used due to lack of surface Ig.
‡ N = 11. Data previously published in detail (20).
§ Numbers represent percentage of cells positive for each marker.

TABLE II

Clinical and Laboratory Features of Patients with Variable Immunodeficiency and Panhypogammaglobulinemia

Patient	Sex	Age	Onset*	Blood lymphocytes/ mm³	Serum			Cellular immunity§	Clinical features
					IgG‡	IgM	IgA		
		yr			*mg/ml*				
Type I									
Per	M	52	24	7,500	2.00	0.10	0.08	Intact	Recurrent sinopulmonary infections, malabsorption, arthralgias, herpes zoster
Min	F	47	35	1,350	4.40	0	0.02	Impaired	Recurrent pneumonia, bronchiectasis
Sch	F	52	49	1,310	0.45	0.58	0	Impaired	Recurrent sinopulmonary infections, pernicious anemia, hepatitis
Phi	F	39	28	470	1.08	0.13	0.05	Intact	Recurrent sinopulmonary infections, splenomegaly, hepatitis, diarrhea, temporal lobe seizures
Roz	M	10	<1	1,230	2.00	0	0	Intact	Recurrent bronchitis, diarrhea, generalized vaccinia in infancy, grand mal seizures
Wal	F	47	28	930	0.85	0	0	Intact	Recurrent pneumonia, bronchiectasis, arthralgias, Giardia infection
Type II									
Sco	F	16	<1	5,600	2.80	0.10	0.27	Impaired	Recurrent sinopulmonary infections, arthritis, dermatitis, aseptic necrosis hip
McK	F	37	18	2,670	0.25	0.05	0.06	Impaired	Recurrent pneumonia, bronchiectasis, hepatosplenomegaly
Pas	M	10	4	2,300	0.60	0	0	Intact	Recurrent sinopulmonary infections, meningitis
Cat	F	40	5	780	0	0	0	Intact	Mild recurrent sinopulmonary infections, rheumatoid arthritis
Type III									
Ste	M	28	21	2,550	0.62	0	0	Intact	Recurrent sinopulmonary infections, malabsorption, nodular lymphoid hyperplasia, Giardia infection
Wag	M	15	7	780	0.22	0.12	0	Intact	Recurrent sinopulmonary infections, malabsorption, dermatitis
Kil	M	18	2	1,515	1.50	0	0	Impaired	Recurrent sinopulmonary infections, bronchiectasis, diarrhea, monoarticular arthritis. Received transfer factor prior to study
Bad	M	28	<1	3,200	1.45	0	0	Intact	Recurrent respiratory infection, bronchiectasis
Type IV									
And	F	62	43	1,090	1.38	0.17	0.12	Intact	Chronic bronchitis, demyelinating disease (?), hypothyroidism, inactive tuberculosis

* Age at onset of recurrent infections. In several cases hypogammaglobulinemia was diagnosed months or years later.

‡ All patients except McK, Cat, and And were receiving parenteral gamma globulin.

§ Intact: delayed hypersensitivity skin reactions to test antigens (see Methods). Impaired: absence of delayed hypersensitivity skin reactions including sensitization to dinitrochlorobenzene, and diminished (compared with normal controls) or absent response of PBL to stimulation by phytohemagglutinin.

trast microscopy. Lymphocytes were considered E rosette positive if any SRBC were adherent. Approximately 90% of E rosettes involved three or more SRBC.

In certain cases B and T cell markers were also evaluated simultaneously on individual cells. This was accomplished by first staining the PBL with the appropriate fluorescent reagent, washing thoroughly, and then applying the rosetting technique. Individual cells were then viewed alternately under phase contrast and fluorescent illumination and scored for the presence of one, both, or neither marker.

RESULTS

The series of patients with variable immunodeficiency and panhypogammaglobulinemia under study here showed heterogeneity with regard to clinical presentation. Of the 15 patients, seven were male and eight female; seven had onset of disease early in life while eight had onset postpuberty; in nine patients cellular immunity was intact while in six it was impaired. When lymphocytes from these patients were evaluated for surface Ig, aggregated Ig binding, and E rosette formation, four distinct patterns were observed which have arbitrarily been designated types I–IV. The results of studies with lymphocyte surface markers are presented in Table I, and the clinical and laboratory features of the patients are listed in Table II.

Type I. 6 of 15 patients were observed to have percentages of B lymphocytes within the normal range as measured by both surface Ig staining and aggregated Ig binding. These two markers have previously been shown to be independent (8–10). The T lymphocyte marker, E rosettes, showed good agreement (inverse correlation) with the B lymphocyte markers, and addition of the percentages of B and T cells accounted for almost 100% of the lymphocytes. Five of the six patients in this group had late onset of their illness and all six patients had severe illness as evidenced by structural lung changes secondary to infection.

Type II. Four patients showed near absence of surface Ig-bearing lymphocytes but low (one patient) or normal (three patients) percentages of aggregated Ig-binding cells. Further, an inverse correlation was observed between percentages of cells binding aggregated Ig and those forming E rosettes. These observations suggested the presence in these patients of B lymphocytes lacking easily detectable surface Ig. It seemed important in these cases to obtain direct evidence that aggregated Ig was binding only to B lymphocytes. Therefore, double labeling studies (aggregated Ig binding + E rosettes) were performed with PBL from three of these patients (Sci, Mck, Cat). Nearly all the cells binding aggregated Ig did not form E rosettes. Previous studies have shown that these markers do not interfere with each other when evaluated simultaneously on individual cells (20). Thus, it appeared that aggregated Ig binding was a reliable marker for B cells whether surface Ig was detectable or not, and further evidence was obtained that abnormal B lymphocytes were present in these patients. Three of the four patients were female, and two of these women and the one male in this group had early onset of their illness.

Type III. Four of the 15 patients showed low (three patients) or low normal (one patient) percentages of B cells as evaluated by both B lymphocyte markers and normal percentages of T cells, but the total number of lymphocytes accounted for by addition of B and T cell markers (80.0–88.7%) was below the lower limit of the normal range (89.0%) and substantially below the mean totals of B and T cells in normal donors (96.7–97.7%). Thus, in these four patients, from 11.3 to 20.0% of the PBL bore no markers ("null" cells) as compared with a mean of 2.3–3.3% in normal individuals.

A variety of experiments were performed in an attempt to identify the null cells in these patients. No additional B cells were detected when surface Ig staining and aggregated Ig binding were evaluated by indirect or "sandwich" techniques, which had shown on increased sensitivity in the detection of B cells in certain cases of chronic lymphocytic leukemia (9). Evaluation of B lymphocytes by another independent marker, the receptor for the C3 component of complement (21) (methodology and reagents kindly provided by Dr. Ethan M. Shevach) (22), detected fewer B lymphocytes in these patients than did surface Ig staining or aggregated Ig binding. Modifications of the E rosette technique including use of human AB serum (13) and prolonged incubation between PBL and SRBC did not increase the percentages of T cells detected. These results suggested that the observation of null cells in these patients was not an artifact. All four patients in this group were male and three of four had early onset of their disease.

Type IV. One patient was observed to have high normal percentages of B cells and normal percentages of T cells, but addition of B and T lymphocyte marker percentages totaled considerably more than 100% (114.5–117.2%). It was apparent that some cells bore both B and T cell markers. This was confirmed by double labeling studies. Surface Ig + E rosettes and aggregated Ig binding + E rosettes showed 22.0% and 18.0% double marker cells, respectively. Almost all the double marker cells bound > 3 SRBC. Double marker cells have been observed in all normal donors studied by double labeling techniques but in much lower percentages: mean 3.6%, range 1.0–6.0% (20). Attempts to remove (by trypsin digestion) and then regenerate surface Ig in both normal and this patient's PBL have been unsuccessful in the present study and therefore direct evaluation of whether the double marker population represents T cells with the ability to adsorb Ig has not been possible. However, this patient was not receiving exogenous immuno-

TABLE III

Proposed Classification of Variable Immunodeficiency with Panhypogammaglobulinemia

Type	Pattern observed with B and T lymphocyte surface markers*	Hypothesized defect	No. of patients
I	Normal percentages of B lymphocytes	B lymphocytes unable to differentiate into immunoglobulin-secreting plasma cells	6
II	B lymphocytes lacking easily detectable surface Ig	B lymphocytes unable to synthesize or transport immunoglobulin	4
III	Large subpopulation of "null" lymphocytes	Precursor cells unable to differentiate into B lymphocytes or defective B lymphocytes which lack identifiable markers	4
IV	Large subpopulation of lymphocytes bearing both B and T cell markers	Activated T lymphocytes secondary to disordered immune response or multipotential precursor cells unable to differentiate into B lymphocytes	1
V	All T lymphocytes	Complete absence of B lymphocytes and precursors	0

* Evaluation of peripheral blood lymphocytes for surface Ig, aggregated Ig binding, and E rosette formation.

globulin and it therefore seemed unlikely that double marker cells were related to antiimmunoglobulin activity.

DISCUSSION

The present observations show that it is possible to separate patients with variable immunodeficiency and panhypogammaglobulinemia into at least four distinct types by evaluation of their PBL for surface Ig and aggregated Ig binding (independent B lymphocyte markers), and E rosette formation (a T lymphocyte marker). These data provide the basis for a proposed classification (Table III) for this clinically heterogenous group of patients with primary immunodeficiency.

Six patients were observed to have percentages of B lymphocytes within the normal range as directly quantitated by two independent B cell markers (surface Ig staining and aggregated Ig binding) and indirectly by inverse correlation with a T cell marker (E rosettes). This group has been designated type I. The findings in these six patients confirm and extend the observation by other laboratories that some patients with variable immunodeficiency have normal numbers of surface Ig-bearing PBL (15–18). In view of the fact that these six patients were extremely deficient in serum immunoglobulins it seems apparent that their circulating B lymphocytes are not necessarily functional effector cells. Since such patients have been shown to lack Ig-secreting plasma cells (15, 17, 18), this suggests that the defect is an inability of their B lymphocytes to differentiate into Ig-secreting plasma cells. This hypothesis is based on the assumption that the Ig on the lymphocytes of such patients is endogenously produced by the B cell upon which it appears rather than absorbed from the serum. Evidence for this remains indirect at present and is based on the detection of IgM- and IgA-bearing lymphocytes in patients with variable immunodeficiency whose serum lacked detectable amounts of these two Ig classes (15, 17, 18), and on evidence that PBL from patients with variable immunodeficiency can synthesize Ig (23). On the other hand, a single immunodeficient patient has been described whose B cells appeared to bear Ig which was not endogenously produced (24). Therefore, membrane Ig turnover studies will be important in the six type I patients to establish the endogenous or exogenous nature of the lymphocyte surface Ig.

One of the type I patients (Phi) was lymphopenic, and thus while this patient had a normal percentage of B cells, the total number of B lymphocytes was decreased. Since this patient did have some B lymphocytes and since the absolute number of B cells necessary for normal humoral responses is unknown, this patient has been included as a type I. Nevertheless, the possibility exists that the defect in this patient may be related to the diminished number of B cells rather than a functional defect of the B lymphocytes which are present.

The peripheral blood of four patients contained a subpopulation of lymphocytes which could be identified as B cells by their ability to bind aggregated Ig and failure to form E rosettes, but which lacked fluorescence-detectable surface Ig (type II). While monocytes have

an Fc receptor (25), do not form E rosettes (11, 13, 14), and may occasionally resemble lymphocytes morphologically, it seemed unlikely that these cells were monocytes for several reasons. In addition to morphologic criteria these cells did not adhere to nylon fiber and did not phagocytize (both properties of monocytes). Furthermore, monocytes are surface Ig positive apparently due to the presence on their surface membrane of adsorbed cytophilic IgG (18). Peripheral blood monocytes evaluated in the two type II patients receiving parenteral gamma globulin (Sco and Pas) were positive for surface Ig.[3] Thus the Ig-negative, E rosette-negative, aggregated Ig-binding cells appear to be B cells, and the defect in this group of patients seems to be an inability of these B lymphocytes to develop surface Ig. Such cells would presumably be incapable of recognizing and responding to antigens. Circulating B lymphocytes lacking easily detectable surface Ig, similar to those seen in these four patients, have been reported in a patient with severe hypogammaglobulinemia and thymoma (24). That patient's B lymphocytes, when cultured in media supplemented with fetal calf or normal human serum, developed detectable surface Ig. In contrast, no evidence for Ig synthesis was obtained in similar experiments with the cells of one of the type II patients (Mck).[4] This suggests that the development of surface Ig on B lymphocytes can be blocked at more than one level. This block might be an inability to synthesize Ig or, if synthesized, to transport it to the surface membrane. It should be possible to differentiate a defect in synthesis from a block in transport by analysis of these patient's B cells for cytoplasmic Ig.

Four patients (type III) were observed to have low (low normal in one case) percentages of B cells and, in addition, a substantial subpopulation (11.3–20.0% compared with means of 2.3–3.3% in normal individuals) of lymphocytes which apparently bore neither B nor T cell markers (null cells). The nature of these null cells and the level of the defect in these patients is not established by the present study. Null cells could represent a population of B lymphocyte precursors which are unable to further differentiate. There is some evidence in the mouse that theta-negative, Ig-negative lymphocytes are members of the B lymphocyte line (26, 27). Alternatively, null cells could be defective B cells which lack identifiable markers. It is of interest that all four type III patients were male. Studies of B and T lymphocytes in members of their families may shed additional light on the nature of the defect and the possible role of genetic factors in the pathogenesis of the disease.

One case was characterized by the presence of a subpopulation (18.0–22.0%) of circulating lymphocytes which bore both B and T cell markers (type IV). In contrast, double marker lymphocytes are present in only small numbers (3.6% range: 1.0–6.0%) in the peripheral blood of normal man (20). The nature of cells bearing both B and T cell markers remains unclear, and the relationship of a markedly increased population of such cells to the immunodeficiency state in this patient is unknown. Recent reports have presented evidence that some T lymphocytes can adsorb cytophilic antibody (28), and that antigen activated T cells can bind antigen-antibody complexes (29) and bear easily detectable surface Ig (30). If double marker cells prove to be activated T cells, then the large population of such cells in this patient could be a manifestation of a disordered immune response (e.g. an autoimmune phenomena) which results in inhibition or loss of Ig synthesis. Alternatively, double marker lymphocytes may represent a population of multipotential precursor cells which are able to differentiate into B or T cells. If this were the case, this patient's defect could be related to a block in the differentiation of such cells. However, this possibility is hypothetical and in opposition to current concepts of lymphoid differentiation.

Among the 15 patients, none were observed in whose peripheral blood only T cells were enumerated. Such a patient would presumably lack all elements of the B cell line. This pattern has been designated type V in the expectation that some such patients will be encountered.

It is important to note that while no change in the pattern of markers was observed in individual patients over weeks or months, this does not rule out the possibility of such a shift over longer periods. Follow-up studies are in progress to document the occurrence or absence of such shifts.

The observations presented and the classification proposed represent a further step towards delineation of the group of patients with variable immunodeficiency into homogenous syndromes. It is anticipated that application of these techniques to evaluate lymphocyte populations in the lymphoid organs of these patients, during infections, and in family studies, as well as the use of other markers and techniques, will provide information necessitating the modification or alteration of this classification.

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