PRE-B CELLS AND OTHER POSSIBLE PRECURSOR LYMPHOID CELL LINES DERIVED FROM PATIENTS WITH X-LINKED AGAMMAGLOBULINEMIA*

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X-linked agammaglobulinemia is characterized in the majority of the cases by the absence of lymphocytes bearing surface immunoglobulin (sIg^+)[1] from peripheral blood, in addition to a marked decrease of serum Ig levels (1–3). Recently, pre-B cells and rare sIg^+ B cells have been demonstrated in the bone marrow of these patients (4). In contrast, circulating sIg^+ lymphocytes are present in most cases of severe combined immunodeficiency (SCID) and common variable immunodeficiency (CVID) (4–7).

Epstein-Barr virus (EBV)-transformed lymphoblastoid lines have been obtained readily in our laboratory from peripheral blood of normal individuals as well as SCID and CVID patients. Repeated attempts to obtain EBV-transformed cell lines from the blood of those with X-linked agammaglobulinemia have not been successful. However, in the present study, multiple EBV-transformed cell lines were established from the bone marrow of three patients. Analysis of immunoglobulin synthesis indicated most of these lines differed markedly from those derived from normal individuals, with some resembling pre-B cells, and others possible precursor cells of the B cell lineage. Preliminary results have been presented previously (8).

Materials and Methods

Patients. Six cases of X-linked agammaglobulinemia were studied. The criteria for the diagnosis included severely depressed Ig levels, lack of sIg^+ cells in the circulation, absence of specific antibodies with normal in vitro T cell responses in the mixed lymphocyte reaction and to phytohemagglutinin and concanavalin A, a clinical history of early onset of infection, and involvement of other male relatives in all cases except patient Ric., from whom no definite family history could be elicited. In addition, two sisters with a syndrome indistinguishable from X-linked agammaglobulinemia (9) were also included. Seven patients with CVID and six patients with SCID were studied.

Cell Cultures and Initiation of Cell Lines. Cell cultures and initiation of cell lines were carried out as described previously (10). Mononuclear cells isolated by Ficoll-Hypaque gradient centrifugation were depleted of T cells by the sheep erythrocyte rosetting method. The non-T cells were then used in the initiation of cell lines. In certain cases, the non-T cells were depleted

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[1] CVID, common variable immunodeficiency, EA_{IgG}, ox erythrocytes coated with IgG antibodies; EAC_m, ox erythrocytes coated with IgM antibodies and mouse complement; EBNA, Epstein-Barr viral nuclear antigen; EBV, Epstein-Barr virus; SDS, sodium dodecyl sulfate; mRNA, messenger RNA; SCID, severe combined immunodeficiency; sIg, surface immunoglobulin.

of monocytes either by the removal of iron-ingesting cells with a magnet or by the removal of adherent cells.

Immunofluorescence and Methods for Detection of Ig Synthesis. Detection of sIg^+ and intracellular Ig and Ia antigens by immunofluorescence was carried out as described (10). For internal labeling, cells (9×10^6/ml) were incubated with 20 μCi/ml [^{35}S]methionine (700 Ci/mmol, New England Nuclear Inc., Boston, Mass.) in methionine-free RPMI-1640 medium that contained 5% dialyzed agammaglobulinemic horse serum (Grand Island Biological Co., Grand Island, N. Y.) for 4 h at 37°C in a 5% CO_2 humidified atmosphere. After three washes, the cells were lysed in a buffer with 1% Triton X-100 precipitated with either affinity column-purified anti-μ or anti-light-chain antibodies, and processed for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as previously described (11).

Fc Receptors, C3 Receptors, and Other Methods. Fc receptors and C3 receptors were detected by a rosette formation method with either ox erythrocytes coated with IgG antibodies or IgM antibodies plus mouse complement, respectively, as described previously (12). The detection of Epstein-Barr viral nuclear antigen (EBNA) was performed as described by Reedman and Klein (13).

Results

Cell Lines from SCID and CVID Patients. The non-T fraction of the peripheral blood from seven patients with CVID and six patients with SCID was incubated with EBV-containing supernates from cell line B95-8. Within 2–4 wk, cell lines were established. Morphologically, these cell lines resembled those obtained from normal individuals. They were pleomorphic and grew as clumps in suspension. All these lines were shown to be EBNA positive and thus contained the EBV genome. Immunofluorescence analyses demonstrated that all of these lines, with one exception, synthesized both membrance and cytoplasmic Ig. The exceptional line had no detectable cytoplasmic or membrane Ig and was from an SCID patient in whom no circulating sIg^+ cells were present.

Cell Lines from Patients with X-linked Agammaglobulinemia. Repeated attempts to derive cell lines with EBV preparations from the peripheral blood of six patients with X-linked agammaglobulinemia and two females with a syndrome clinically indistinguishable from the X-linked cases were not successful. Depletion of monocytes and adherent cells from the non-T fraction did not help. The ages of these patients were 8 mo (Josh), 20 yr (Ric), 21 yr (Bry), and 28 yr (Bro).

Bone marrow aspirates were obtained from four of these patients. 11 cell lines were obtained from three of them. No cell lines were obtained from the oldest patient, Bro. In general, it took 4–6 wk to establish these lines. Except for Josh 7, supernates that contained EBV from cell line B95-8 were used. Josh 7 was established without the aid of exogenous EBV and was established 10 wk after bone marrow cells were in culture. 10 of the 11 cell lines had growth characteristics indistinguishable from the common lymphoblastoid lines. Josh 7 was unusual in that the cells were uniform and round. This type of unusual round cell line has been reported previously (10).

Ig Synthesis by Cell Lines from Patients with X-Linked Agammaglobulinemia. Either a lack of Ig expression or unusual Ig patterns were encountered in some of these lines when immunofluorescence analysis was carried out (Table I). Josh 2 resembled typical normal lines and expressed both membrane IgM and cytoplasmic IgM with λ light chain. Both μ and λ determinants were detected in the supernate. Josh 1 and Josh 5 had few Ig-containing cells, although none of the cells had sIg. In the case of Josh 4, only cytoplasmic μ chain was detected. The presence of only μ chain was also found in the supernate of Josh 4 by both hemagglutination inhibition and immunoprecipi-

TABLE I

Percentage of Cells in Different Cell Lines from Patients with X-Linked Agammaglobulinemia Showing Membrane Ig and Cytoplasmic Ig as Detected by Fluorescence with Ig Antisera

Cell lines	Membrane Ig					Cytoplasmic Ig				
	IgM	IgG	IgA	κ	λ	IgM	IgG	IgA	κ	λ
			%					%		
Josh										
1	0	0	0	0	0	0*	0*	0	0	0*
2	18	0	0	0	18	33	0	0	0	37
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	47	0	0	0	0
5	0	0	0	0	0	1	0	0	2	2
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
Ric										
1	0	0	0	0	0	45	0	0	0‡	0
2	0	0	0	0	0	40	0	0	0	0§
Bry										
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0

* Indicates occasional cells stained brightly, and could be identified as plasma cells.

‡ 20% of the cells stained dully and definitely above background staining, although the staining was much less intense than that for IgM.

§ 28% of the cells stained dully as those in Ric I.

tation of [^{35}S]methionine-labeled chain identified by SDS gel electrophoresis. In the two lines from patient Ric, large numbers of brightly stained cells were observed with anti-μ antibodies. This contrasted with faint staining of lesser numbers of cells by the anti-light chain reagents. This finding suggested the synthesis of small amounts of light chain and was confirmed by biosynthetic studies involving [^{35}S]methionine and gel electrophoresis. Of five cell lines in which no Ig expression was shown by fluorescence, three (Josh 6, Bry 1, and Bry 2) were shown to make detectable amounts of heavy and light chains by biosynthetic studies. However, repeated experiments with [^{35}S]methionine labeling failed to reveal any Ig synthesis in either Josh 3 or Josh 7.

The following represent typical findings in the biosynthetic studies. Fig. 1 shows the Ig biosynthetic patterns of three cell lines from patient Josh. Anti-μ antibodies brought down both μ and light chains from Josh 2. In the case of Josh 3, no Ig chain was precipitated. From Josh 4, only labeled μ chain was detected. To exclude the possibility that this chain existed in combination with unlabeled light chain formed prior to the [^{35}S]methionine pulse, anti-light-chain antibodies with both κ and λ specificities were used as the precipitating antibodies. These anti-light-chain antibodies brought down both μ and L chains from Josh 2, but no Ig chains from either Josh 3 or Josh 4, thus confirming the absence of light chain in Josh 4.

Fc Receptors, C3 Receptors, and Other Studies. Ox erythrocytes coated with isolated IgG antibodies (EA_{IgG}) were used to detect Fc receptors on the surface of cells. Except for Josh 7, 15–30% of cells formed EA_{IgG} rosettes with eight cell lines from two individuals (Table II). In the case of Josh 7, 81% formed EA_{IgG} rosettes. For C3 receptors, EA_{ox} coated with IgM antibodies were incubated with a C5-deficient mouse

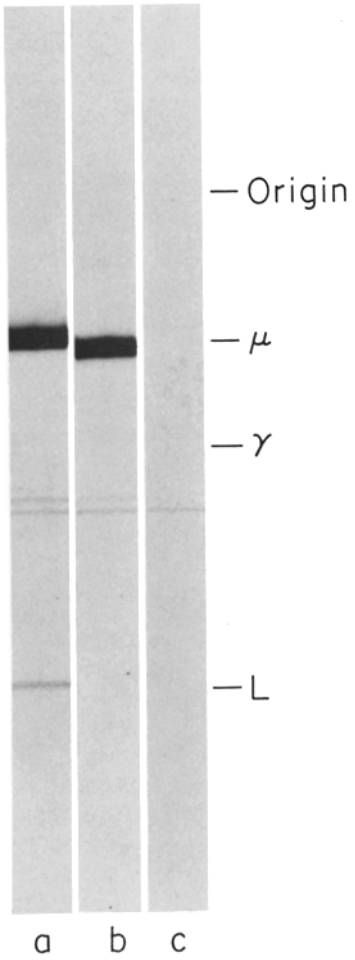

FIG. 1. Ig synthesis by three cell lines from a patient with X-linked agammaglobulinemia. Cells were labeled with [^{35}S]methionine. Triton X-100 lysates were precipitated with anti-μ antibodies. The immunoprecipitates were reduced and alkylated and analyzed by electrophoresis in 7–15% gradient SDS-polyacrylamide gel. (a) cell line Josh 2; (b) cell line Josh 4; and (c) cell line Josh 3.

serum to generate EAC_m. 83% of cells in Josh 7 formed EAC_m rosettes, and 14–56% of cells formed EAC_m rosettes in the remaining cell line. In all 11 cell lines, >95% of the cells stained for Ia antigens and were positive for the EBNA; spontaneous sheep E rosettes were either absent or very rare.

Discussion

Six major types of cell lines were derived from the bone marrow of three patients with X-linked agammaglobulinemia. The first type (Josh 7) was unique in that its cells were round and uniform in shape. Very high percentages of the cells formed EA_{IgG} and EAC_m rosettes. This line showed a complete absence of Ig expression and belongs to a group of unusual EBV that contained round cell lines reported previously (12). The second type (represented by Josh 3) also completely lacks Ig synthesis. However, it cannot be distinguished from the common B lymphoblastoid lines in cell

TABLE II

Expression of Fc and C3 Receptors by Cell Lines from Patients with X-Linked Agammaglobulinemia

Cell lines	Fc receptor (EA_{IgG} rosette-forming cells)*	C3 receptor (EAC_m rosette-forming cells)‡
	%	%
Josh		
1	28	53
2	19	47
3	20	14
4	30	56
5	15	46
6	18	50
7	81	83
Ric		
1	22	33
2	18	48

* Fc receptors on cells were detected by a rosette formation method with ox erythrocytes coated with IgG antibodies. 5–60% of the cells in common EBV-transformed cell lines from normal individuals and patients with other conditions formed EA_{IgG} rosettes.

‡ C3 receptors were detected by a rosette formation method with ox erythrocytes coated with IgM antibodies and mouse complement. 30–50% of the cells in common EBV-transformed cell lines from other individuals formed EACm rosettes.

morphology, growth characteristics, and percentages of EA_{IgG} and EAC_m rosette-forming cells. These two cell lines may represent transformation of B precursor cells at a stage before the expression of Ig. The third type (Josh 4) shows an Ig synthetic pattern typical of pre-B cells (14, 15), in that only cytoplasmic μ chain was made. The fourth type (Ric 1 and 2) express predominantly cytoplasmic μ chain with some cytoplasmic light-chain synthesis. This pattern of Ig synthesis resembles that of Nalm-6-M1, a leukemic cell line representing malignant transformation of B cells at a late pre-B cell stage (16). The fifth type (Josh 1, Josh 5, Josh 6, Bry 1, and Bry 2) showed synthesis of small amounts of both heavy and light chains without the expression of membrane Ig. The sixth type (Josh 2) expresses both membrane and cytoplasmic μ and L chains, and resembles the normal lymphoblastoid lines.

Extensive experience has accumulated in our laboratory in establishing lymphoblastoid lines from both normal individuals and patients with various disorders. Over 100 cell lines from peripheral blood and 15 cell lines from normal bone marrow aspirates have been analyzed by immunofluorescence, and they were uniformly found to synthesize both membrane and cytoplasmic Ig. Thus, the patterns of Ig synthesis by most of the cell lines from X-linked immunodeficient patients were quite unique.

The presence of EBNA indicates that these cell lines contain EBV genome. It suggests that receptors for EBV are present on some if not all B cells at developmental stages before sIg synthesis. Thus, like the Abelson virus in the mouse, EBV can infect B cells at various stages of development (15). This hypothesis is being tested by transformation of non-T populations depleted of sIg^+ cells from normal individuals.

The isolation of cell lines from patients with X-linked agammaglobulinemia was initially undertaken in an attempt to obtain cells in culture that reflected the in vivo

defect in Ig synthesis. However, it is clear that at least certain of the cell lines described above were of the normal precursor type. This is especially evident from the various pre-B cell lines. It is also evident from the round cell type now obtained from individuals with normal Ig synthesis. Our concept at the present time is that the other unique cell lines, those without any Ig synthesis, and those with very little Ig, may also represent precursor cell types. It became clear in this study that the absence of mature B cells in these patients played a major role in the isolation of the unique types; ordinarily, the mature B cells would be transformed and overgrow the culture system. Thus, it would appear that these cases represent a fertile source of material for studying B cell ontogeny. However, the possibility that some of the cell lines reflect abnormal Ig synthesis characteristic of the disease has not been ruled out. It seems more likely, as has been proposed before (4), that the disease results from a failure of terminal differentiation of normal precursor cells.

It has become apparent that these cell lines are of considerable value for the study of precursor B cells. For example, it has been demonstrated that two distinct translation products of human μ chain representing the heavy chain of membrane-bound and secreted IgM were in cells of B lymphoblastoid lines as well as in mature B cells (11). Results involving in vitro translation of extracted messenger RNA (mRNA) from Josh 4 indicate the presence of two species of μ chain mRNA and the absence of light chain mRNA. Experiments are in progress to determine whether these two μ chains are analogous to those found in the more mature B cells. If this proves to be the case, it suggests that light-chain expression may play an important role in determining membrane μ chain transport to the cell surface. In addition, these lines should prove a valuable source for the isolation of DNA in order to study the nature of the genes coding for Ig during the early period of human B cell development.

The establishment of EBV-transformed lymphoblastoid lines of the normal type from peripheral blood of patients with CVID and SCID agrees with the results reported by other investigators (17–19). The lack of success in obtaining B lymphoblastoid lines from peripheral blood of patients with X-linked agammaglobulinemia is also in agreement with a previous report that, only in rare patients of X-linked agammaglobulinemia with circulating sIg^+ cells, EBV-transformed lymphoblastoid lines were established (18). Recently, successful establishment of cell lines that expressed both sIg and cytoplasmic Ig from peripheral blood of the common type of patients was reported (19). Finally, the fact that more cell lines were established from the bone marrow of the youngest patient (Josh) in the present report suggests that there may be more precursor B cells and some mature sIg^+ B cells in the bone marrow of patients with this disorder at an early age. There is a decline of these precursor B cells as the patients grow older. The mechanism for this decline is not readily apparent. However, further documentation of this impression is needed.

Summary

A group of unique Epstein-Barr virus-containing cell lines was derived from the bone marrow of three patients with X-linked agammaglobulinemia. Efforts to obtain cell lines from the peripheral blood of these patients were uniformly unsuccessful. Immunofluorescence analyses as well as biosynthetic studies with [^{35}S]methionine indicated unusual patterns of Ig synthesis in many of these bone marrow derived lines. Seven of the lines were of particular interest in that two produced no Ig of any

type; two others showed no Ig by fluorescence but small amounts by [^{35}S]methionine labeling; one expressed only cytoplasmic μ chains without any evidence of light chain synthesis, and two produced primarily μ chains with only slight amounts of light chains. One of the lines without membrane or cytoplasmic Ig studied in detail grew like a typical lymphoid line and was carried in intermittent culture over a period of 2 yr without Ig expression. One line grew quite differently and resembled the round cell type described previously, which has been obtained from a variety of sources. The cell line with cytoplasmic μ chains and no light-chain expression had the characteristic properties of pre-B cells. Three normal type Ig-producing cell lines also were obtained from the patients.

The accumulated evidence obtained in the present study indicates that these unusual cell lines represent normal precursor cells of the B-cell lineage; these grew out in these cases because of the virtual absence of mature B cells that ordinarily overgrow the culture system. However, the possibility that in certain instances they reflect abnormal Ig synthesis characteristic of the disease has not been ruled out.

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