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ECTO-5'-NUCLEOTIDASE ACTIVITY IN LYMPHOBLASTOID CELL LINES DERIVED FROM HETEROZYGOTES FOR CONGENITAL X-LINKED AGAMMAGLOBULINEMIA[1]

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Lymphoblastoid cell lines were established from female relatives of patients with congenital X-linked agammaglobulinemia by Epstein-Barr virus transformation of their peripheral B lymphocytes. Cell lines derived from presumed carriers were characterized by low ecto-5'-nucleotidase activity and a reduced percentage of surface immunoglobulin-bearing cells. Measurement of ecto-5'-nucleotidase activity in newly established lymphoblastoid cell lines may provide a means for the identification of heterozygotes for congenital X-linked agammaglobulinemia.

Patients with congenital X-linked agammaglobulinemia (X-LA)[5] have reduced ecto-5'-nucleotidase (EC 3.1.3.5, ecto-5'-NT) activity in their peripheral blood mononuclear cells (1-3). This reduced activity is the result of both low (~50% of normal) ecto-5'-NT activity in their peripheral T cells (2, 3) and a virtual absence of peripheral B cells (4, 5), which normally have high ecto-5'-NT activity (2, 6). Although the mean ecto-5'-NT activity of B cell preparations from female relatives of X-LA patients was 70% of the value from normal subjects, individual values fell within the range demonstrated by the controls, and we were thus unable to identify which female relatives are carriers of X-LA (2). It is difficult to obtain homogeneous B cell preparations, and assay of mixed cell populations may have contributed to the failure of detecting heterozygotes by this method. We therefore decided to measure ecto-5'-NT activity in lymphoblastoid cell lines derived from potential carriers of X-LA, since these cell lines are established by Epstein-Barr virus (EBV) infection and are solely of B cell lineage (7, 8).

MATERIALS AND METHODS

Cell culture. Lymphoblastoid cell lines were established according to the method of Sly *et al.* (9) from peripheral blood lymphocytes of seven normal subjects and eight female relatives of patients with X-LA. The cells were grown in RPMI 1640 supplemented with 20% fetal calf serum and 3% glutamine in an atmosphere of 5% CO_2 in humidifed air. The cells were maintained at densities of 2 to 12 × 10^5 cells/ml.

Ecto-5'-NT assay. Exponentially-growing lymphoblasts at densities of 8 to 12 × 10^5 cells/ml were washed twice in 40 mM sodium HEPES,[5] pH 7.4, 130 mM NaCl, and 0.4% bovine serum albumin and resuspended in the same buffer at a final density of 5 to 10 × 10^6 cells/ml. The enzyme assay was performed with ^{14}C-IMP[5] (Amersham, Arlington Heights, Ill.) as substrate as previously described for peripheral blood lymphocytes (2). The results are presented as the means of triplicate determinations, which generally agreed within 10%. All assays were performed in the presence and absence of α,β-methylene adenosine 5'-diphosphate (AOPCP, Sigma, St. Louis, Mo.), a specific inhibitor of ecto-5'-NT (10, 11).

Surface immunoglobulin measurements. Cells bearing surface immunoglobulins (sIg) were measured as previously described (12). The results are expressed as the percentage of cells bearing either κ or λ light chains on their surface.

Subject population. Four families with X-LA were studied (see Fig. 1 for pedigree data). In families A, B, and C, the diagnosis of X-LA was based on low serum Ig with a virtual absence of sIg+ peripheral lymphocytes and recurrent bacterial infections in the first months of life. Subjects II.3, III.1, and III.2 of family D also display classic symptoms of X-LA, but subject II.4 has a milder form of the disease with low but identifiable sIg+ lymphocytes, normal concentrations of serum IgM and IgA and depressed IgG. All four affected males in this family also have an isolated growth hormone deficiency. This family has been previously described (13).

RESULTS

Ecto-5'-NT activity in lymphoblastoid cell lines. Ecto-5'-NT activity was measured in newly established lymphoblastoid cell lines as soon as a culture of approximately 5 × 10^6 logarithmically growing cells was established (49 to 120 days after the addition of EBV). In spite of one cell line among the controls with initial high ecto-5'-NT activity that took 120 days to

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[5] Abbreviations used in this paper: X-LA, congenital X-linked agammaglobulinemia; ecto-5'-NT, ecto-5'-nucleotidase; EBV, Epstein-Barr virus; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; IMP, inosine-5'-monophosphate; AOPCP, α,β-methyleneadenosine-5'-diphosphate; sIg+, surface immunoglobulin-positive.

establish, there was little correlation between the length of time needed for the establishment of the culture and the initial ecto-5′-NT activity (Table I). The initial ecto-5′-NT activity was similar in cell lines established from control males and females. There was no difference in the mean length of time necessary to establish the cultures among the control subjects, presumed heterozygotes, and presumed nonheterozygotes (Table I). At the time of the initial assay, ecto-5′-NT activity was markedly deficient in the lymphoblastoid cell lines established from the only maternal grandmother (A.I.1), three of the four mothers (A.II.1, C.I.1, D.II.2), and two of the four sisters (A.III.2, C.II.4) of X-LA patients. We propose that these cell lines are derived from heterozygous carriers of X-LA. The mother whose lymphoblast line showed normal ecto-5′-NT activity (B.I.1) is from a family with only one affected child (B.II.2) and no previous family history of X-LA. Thus, the affected male in this family may represent the expression of a new mutation for X-LA. The sister (B.II.1) of the affected male in this family also appears not to be a carrier, which is consistent with this hypothesis.

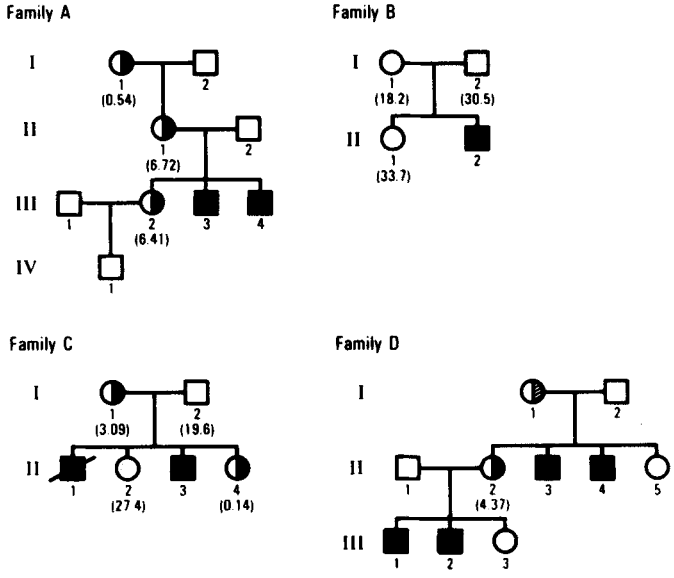

Figure 1. Pedigree analysis of four families with congenital X-linked agammaglobulinemia. ○, □, normal; ◑, presumed heterozygote by measurement of ecto-5′-nucleotidase activity in newly established lymphoblastoid cell line; ⊖, presumed heterozygote by pedigree analysis; and ■, congenital X-linked agammaglobulinemia. AOPCP-inhibitable ecto-5′-nucleotidase activity in newly established lymphoblastoid lines is indicated in parentheses in nmoles/hr/10^6 cells.

During the 5-week period immediately after the initial ecto-5′-NT assays, the cell lines were reassayed at 5- to 7-day intervals. The ecto-5′-NT activities in the lymphoblast lines of five of the six presumed X-LA heterozygotes gradually increased until they reached control values (Fig. 2). This increase in ecto-5′-NT activity was accompanied by an increase in the growth rate of the cultures, with a mean decrease in doubling time of 41%. The ecto-5′-NT activity in a representative control lymphoblast line over the same time period is shown for comparison and is similar to the results obtained for all cell lines derived from control subjects or presumed nonheterozygotes.

Surface immunoglobulin analysis of lymphoblastoid cell lines. The cells from 10 of the above lymphoblastoid lines were examined for the presence of sIg soon after establishment and were also assayed for ecto-5′-NT activity on the same day (Table II). Surface immunoglobulin was expressed on 30, 52, and 86% of the cells from three control lymphoblast lines, and 1.5, 4.0, 26, 32, and 47% of the cells from the lines derived from five presumed heterozygotes. Cell lines derived from the two female relatives of X-LA patients who do not appear to be carriers (B.I.1 and C.II.2) contained 42% and 75% sIg+ cells, and thus appear to be normal with respect to sIg as well as ecto-5′-NT activity. Although the cell line from subject D.II.2, an obligate heterozygote, appears anomalous with the expression of sIg on 47% of the cells, it must be noted that this line had substantial amounts of ecto-5′-NT activity at the time of sIg analysis. Furthermore, although classic X-LA has been

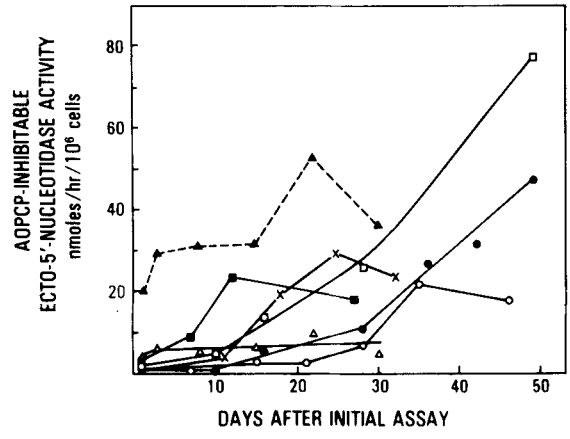

Figure 2. Ecto-5′-nucleotidase activity in newly established lymphoblastoid cell lines as a function of time in culture. ▲, control; X, subject A.I.1; ●, subject A.II.1; ■, subject A.III.2; □, subject C.I.1; ○, subject C.II.4; and △, subject D.II.2.

TABLE I

Ecto-5′-nucleotidase activity in newly established lymphblastoid lines

Fathers and Control Subjects			Female Relatives					
			Presumed heterozygotes			Presumed nonheterozygotes		
Subject	Initial ecto-5′-NT[a]	Days[b]	Subject	Initial ecto-5′-NT	Days	Subject	Initial ecto-5′-NT	Days
M B.I.2	30.5	69	A.I.1	0.54	74	B.I.1	18.2	88
M C.I.2	19.6	64	A.II.1	6.72	49	B.II.1	33.7	44
F control	20.3	75	A.III.2	6.41	49	C.II.2	27.4	71
F control	70.4	69	C.I.1	3.09	64			
M control	63.7	63	C.II.4	0.14	71			
F control	22.9	77	D.II.2	4.37	75			
F control	91.4	120						
Mean ± S.D.	45.5 ± 29	77 ± 20		3.55 ± 28	67 ± 11		26.4 ± 7.8	68 ± 22

[a] AOPCP-inhibitable ecto-5′-NT activity in nmoles/hr/10^6 cells determined as described in *Materials and Methods.*

[b] Length of time from initiation of culture by EBV infection until time of the first ecto-5′-NT assay.

TABLE II

Simultaneous ecto-5′-nucleotidase activities and surface immunoglobulin analysis of newly established lymphoblastoid cell lines

	Ecto-5′-NT Activity	
	nmoles/hr/10^6 cells[a]	% sIg$^+$ cells[b]
Control subjects		
B.I.2	129	86
C.I.2	24.6	30
Control female	28.3	52
Presumed nonheterozygote female relatives		
B.I.1	61.8	42
C.II.2	40.4	75
Presumed heterozygote female relatives		
A.II.1	6.72	1.5
A.III.2	6.41	32
C.I.1	2.44	26
C.II.4	0.14	4.0
D.II.2	10.5	47

[a] AOPCP-inhibitable ecto-5′-NT activity was measured at the time of sIg analysis as described in *Materials and Methods.*

[b] The percentage of cells bearing κ or λ light chains on their surface.

expressed in three males (D.II.3, D.III.1, and D.III.2) over two generations of this family, a fourth male (D.II.4) has a milder form of the disease with some sIg+ B cells and measurable quantities of serum IgM and IgA. Thus, there may be variable expression of the defective gene in this family, or this family could represent a different disease, especially in view of the growth hormone defect expressed in all four males. Two of the cell lines derived from presumed heterozygotes (A.II.1 and C.II.4) were extremely deficient, with only 1.5% and 4% of the cells displaying sIg. These two lines were chosen for reexamination after an additional 5 weeks in culture when their ecto-5′-NT activities had reached control values. At this point, 66% and 68% of the cells expressed sIg, respectively.

Characterization of ecto-5′-NT activity. Ecto-5′-NT activities from newly established control and heterozygote lymphoblast lines (C.II.4) were characterized with respect to pH optimum and K_M. At a time when the catalytic activity of the presumed heterozygote line was 2% of that seen in the control cell line, the enzyme from both cell lines exhibited essentially the same pH optima (6.9 to 7.0) and K_M (10 to 30 μM, data not shown).

DISCUSSION

At the present time, there is no known method for the identification of carriers of X-LA (14). Presumed heterozygotes for this X-linked disorder cannot be distinguished from normal individuals based on numbers of sIg+ lymphocytes, concentrations of serum Ig, or activities of lymphocyte ecto-5′-NT (2). However, lymphoblastoid cell lines initiated by EBV transformation of B lymphocytes from presumed heterozygotes are markedly deficient in ecto-5′-NT activity soon after the cultures are established. After additional time in culture (3 to 5 weeks), the ecto-5′-NT activity gradually increases to control values, and the growth rate of the culture also increases. The following model is proposed to account for these observations.

Ecto-5′-NT activity appears to be a biochemical marker of B lymphocyte maturation and to develop after the acquisition of sIg (15). Both fetal spleen (unpublished data) and cord blood B lymphocytes (15) are markedly deficient in this enzyme activity. These cells display sIg, but are functionally immature, since newborn infants do not acquire the ability to synthesize substantial quantities of IgG and IgA until they are several months old (16). In X-LA patients, B lymphocyte maturation is blocked at an early stage before the acquisition of sIg (4, 5, 17) and consequently also before the acquisition of ecto-5′-NT activity. According to Lyon's Hypothesis (18, 19), carriers for X-LA should have two populations of B lymphocytes: one with sIg and normal ecto-5′-NT activity, and one that is blocked in maturation and which therefore lacks sIg and ecto-5′-NT activity. The latter population is presumably the same defective cell population found in males with X-LA. Low ecto-5′-NT activity in newly established lymphoblast lines derived from X-LA heterozygotes implies EBV transformation of both types of B lymphocytes. However, the cells lacking ecto-5′-NT activity (although apparently preferentially transformed initially) may subsequently be at a selective disadvantage, since over a period of 3 to 5 weeks, both the growth rate and ecto-5′-NT activity of the cultures increase, as if cells with normal ecto-5′-NT activity gradually overgrow cells with little or no activity. An alternate explanation is the maturation of sIg-, ecto-5′-NT-deficient cells into sIg+, ecto-5′-NT-positive cells under the conditions of cell culture.

Although there is evidence for the existence of pre-B cells in the peripheral circulation of males with X-LA that can be stimulated to mature into Ig-secreting cells in culture (20), other investigators have reported an absence of EBV-receptor bearing lymphocytes in these children (17), and the difficulty of establishing EBV-transformed lymphoblast lines from males with X-LA is widely acknowledged. However, our data are not necessarily in conflict with these earlier reports. We also have been unsuccessful in our attempts to establish EBV-transformed cell lines from X-LA patients. The frequency of sIg-, EBV-receptor+ lymphocytes in X-LA patients may be too low to be detected by the methods previously employed. Alternatively, in the presence of normal sIg+ lymphocytes, the situation that occurs in the heterozygotes, perhaps ecto-5′-NT-negative B lymphocytes may be able to partially overcome their genetically determined maturational block.

If our model for the role of ecto-5′-NT in lymphocyte maturation is correct, then newly established lymphoblast lines from X-LA heterozygotes should be deficient in sIg as well as in ecto-5′-NT activity. Indeed, newly established lymphoblast lines from the presumed heterozygotes had a lower percentage of sIg+ cells when compared with control cell lines; two of the presumed heterozygote cell lines demonstrated a virtual absence of sIg+ cells. When the ecto-5′-NT activity increased to normal values in these two cell lines, the percentage of sIg+ cells also increased 15- to 40-fold. These results are consistent with the overgrowth of ecto-5′-NT-positive cells to displace sIg-negative, ecto-5′-NT-deficient cells, but do not rule out the alternate explanations mentioned above. Although the analysis of sIg supports the concept of an increased percentage of sIg-negative cells in newly established lymphoblast lines from carriers for X-LA, the large variability in the number of cells expressing sIg in control lines makes it impossible to positively identify carriers by this method in every case.

Our model further predicts that the low activity of ecto-5′-NT in patients with X-LA is a result of a block in B cell maturation rather than of a mutation in the structural gene for ecto-5′-NT. Although not conclusive, our pH optima and K_M data are consistent with low numbers of cells with structurally

normal ecto-5′-NT in newly established lymphoblast lines from X-LA heterozygotes.

Lymphoblastoid cell lines from additional control subjects and carriers for X-LA must be established and assayed for ecto-5′-NT activity in order to test the validity of this heterozygote detection test. Experiments are currently in progress to isolate and identify ecto-5′-NT-negative and -positive lymphoblasts by cloning in order to confirm the hypothesis that the lymphoblasts that are deficient in ecto-5′-NT activity are the same ones that are deficient in sIg and to further demonstrate the existence of two B lymphocyte populations in the carriers. Unfortunately, we are not assured of success in isolating ecto-5′-NT-negative clones from the X-LA heterozygotes, in view of the difficulty in establishing lymphoblastoid lines from X-LA patients. However, if such clones can be isolated, they will be a potentially valuable source of cells for investigating the mechanism of immune dysfunction in patients with X-LA.

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