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Lymphocyte 5′-nucleotidase in primary hypogammaglobulinaemia and cord blood

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SUMMARY

The 5′-nucleotidase (5′-N) deficiency on circulating mononuclear cells of patients with 'common variable' hypogammaglobulinaemia (CV-H) was shown to be the result of one or more of three factors: reduced T cell 5′-N activity, a reduced percentage of circulating B cells, and a low B cell 5′-N activity. A lack of circulating B lymphocytes, together with a low T cell 5′-N activity in some cases, was found to be responsible for the deficiency of lymphocyte 5′-N in patients with X-linked hypogammaglobulinaemia (X-H). The low levels of 5′-N activity in CV-H and X-H patients were not due to abnormal compartmentalization of the enzyme, altered enzyme kinetics, or the presence of a regulatory factor.

Cord blood B and T cells have a lower 5′-N activity than adult lymphocytes. The patient and cord blood data are discussed in relation to the stage of cellular maturity.

INTRODUCTION

Most patients with 'common variable' hypogammaglobulinaemia (CV-H) and X-linked hypogammaglobulinaemia (X-H) have low levels of ecto-5′nucleotidase (5′-N) activity on their circulating mononuclear cells (Johnson *et al.*, 1977; Webster *et al.*, 1978; Edwards *et al.*, 1978). We have recently reported that B lymphocytes account for about 40% of the total 5′-N activity of circulating mononuclear cells from healthy subjects (Rowe *et al.*, 1979), which suggests that the low–normal 5′-N activities found in X-H patients (Webster *et al.*, 1978) may result from the lack of circulating B cells. However, the lower activities reported by Edwards *et al.* (1978) indicate that the T cells may also have a reduced 5′-N activity. The situation is more complicated in CV-H patients, some of whom may have normal numbers of circulating B cells (Cooper & Lawton, 1972).

We have studied the 5′-N activities of T and B lymphocytes from cord blood and blood from hypogammaglobulinaemic patients in order to determine whether an absent or immature population was responsible for the low 5′-N levels. The possibility that the decreased 5′-N activity was the result of an altered enzyme, or the presence of a regulatory factor, has also been investigated.

PATIENTS AND METHODS

Subjects. Eight patients with X-linked hypogammaglobulinaemia (X-H) were studied, all with affected male relatives and receiving weekly gammaglobulin injections. Their mean age was 18 years (range 9–28 years). Less than 1% B lymphocytes was found in the circulating mononuclear cells when tested by either fluorescent or rosetting techniques. The mean serum IgG level was 2·7 g/l (range 0·8–3·3). Serum IgA and IgM levels were less than 0·1 g/l.

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Fourteen patients with 'common variable' hypogammaglobulinaemia (CV-H) were studied, ten of whom developed their disease as adults. Their mean age was 39 years (range 19–51). Using the rosetting technique, the mean percentage (± s.d.) of B lymphocytes with surface IgM (sIgM$^+$ cells) found in the circulating mononuclear cells of these patients was 3·9± 2·2 compared with 6·5± 2·7 found in parallel controls. These patients had a mean serum IgG level of 2·5 g/l (range 0·4–4·6). Serum IgA and IgM levels in these patients ranged from < 0·04–0·3 and 0·1–1·8 g/l respectively. These patients were also receiving regular immunoglobulin replacement therapy.

The mean age of the normal healthy volunteers used in this study was 31 years (range 21–68 years), and the normal 95% range of serum immunoglobulin levels found in this laboratory are: IgG, 5·9–17·2 g/l; IgA, 0·5–4·0 g/l; IgM, 0·6–2·0 g/l. Cord blood was obtained from placentae following normal full-term deliveries.

Isolation of cells. Mononuclear cells were obtained from fresh heparinized peripheral blood by Ficoll–Triosil density gradient separation (Böyum, 1968). Pure T and B cell populations were isolated from the mononuclear cells as described previously (Rowe *et al.*, 1979), using a method based on the procedure of Parish *et al.* (1974). Briefly, T cells were rosetted with SRBC and separated on a Ficoll–Metrizoate gradient using a simplified one-step procedure outlined by Gmelig-Meyling & Ballieux (1977). B cells with surface IgM and IgG (sIgM$^+$ sIgG$^+$ cells) were isolated from non-T cells by rosetting with OxRBC coated with goat anti-human IgM and IgG, and separated on a Ficoll–Metrizoate gradient (de Gast & Platts-Mills, 1979). The B cell preparations contained less than 2% contaminating T cells. Both control and purified cell preparations were incubated overnight at 37°C in RPMI medium buffered with bicarbonate and supplemented with 2 mM glutamine, 100 iu/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated foetal calf serum. In some experiments mononuclear cells from patients and normal subjects were mixed 1:1 and cultured at 37°C for 18 hr in the same medium.

Enumeration of B lymphocytes. The percentage of B lymphocytes in patient peripheral blood was determined by immunofluorescence and by rosetting with OxRBC coated with goat anti-human IgM (de Gast & Platts-Mills, 1979).

5′-Nucleotidase assay. Intact cells were washed three times in 10 mM tris-buffered saline (pH 7·5). 10^5–10^6 cells were assayed for 5′-N activity in the presence of 20 μM AMP, 60 μM β-glycerophosphate, and 10 mM $MgCl_2$ at pH 8·5 using a radiochemical method described previously (Rowe *et al.*, 1979).

Determination of the apparent Michaelis constant (Km) for 5′-nucleotidase. The initial rate of reaction (V) of 5′-N on intact circulating mononuclear cells was determined for six substrate concentrations (S), ranging from 5–50 μM AMP. The reaction mixture was essentially the same as that used for the routine 5′-N assay except that the concentration of β-glycerophosphate was raised to 150 μM to ensure efficient inhibition of alkaline phosphatase (Belfield & Goldberg, 1968) at the higher substrate concentrations.

The apparent Km was determined using plots of V against V/S (Eadie, 1942) for which the 'best fit' lines were determined by the method of least squares. The standard error of the Km values was usually about 9%.

Solubilization of cells with detergent. 10^5–10^6 cells in 0·45 ml of 10 mM tris-buffered saline, pH 7·5, were lysed by mixing with 50 μl of 10% w/v triton X-100 detergent. 5′-N activity was assayed as usual in a final volume of 1 ml.

Freeze–thaw disruption of cells. Mononuclear cells were resuspended in 10 mM tris-buffered saline, pH 7·5, at a concentration of 2×10^6 cells/ml, then frozen to −20°C and stored overnight before thawing. Particulate and soluble fractions were obtained by centrifugation at 38,000 *g* for 45 min at 4°C. The particulate fraction was washed in 10 mM tris-buffered H_2O, pH 7·5.

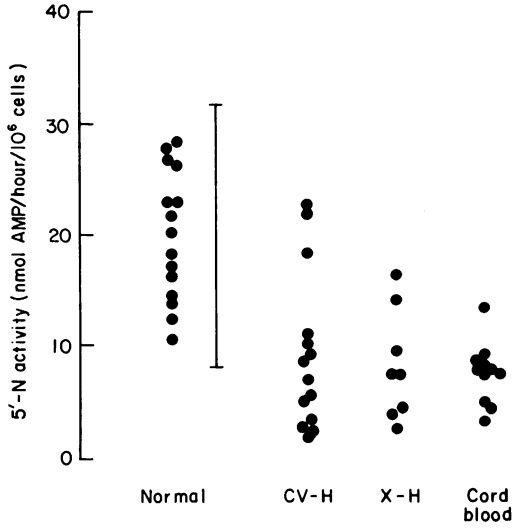

FIG. 1. 5′-Nucleotidase activity on circulating mononuclear cells from healthy adults, patients with CV-H and X-H, and cord blood. The bar represents the normal adult range (mean± 2 s.d.).

TABLE 1. 5′-Nucleotidase activities (nmol AMP/hr/10^6 cells) of blood mononuclear cells

Source of cells	Patient No.	Total mononuclear cells	T cells	B cells (sIg^+)*
Normal (mean ± s.d.)		19·9 ± 5·9 ($n = 15$)	13·5 ± 3·4 ($n = 15$)	54·9 ± 13·8 ($n = 9$)
'Common variable' hypogammaglobulinaemia (CV-H)	1	1·8	2·1	—
	2	2·3	3·1	—
	3	2·8	1·5	23·4
	4	3·6	1·1	n.d.
	5†	5·1	5·7	8·9
	6	5·6	2·7	n.d.
	7	8·5‡	3·9	78·0‡
	8†	9·2‡	5·4	24·9
	9	10·1‡	7·2‡	34·6‡
	10	10·9‡	7·9‡	64·6‡
	11†	18·1‡	10·5‡	—
	12†	21·6‡	20·8‡	17·4
	13	22·3‡	16·8‡	—
X-Linked hypogammaglobulinaemia (X-H)§	1	2·4	4·8	—
	2(a)	3·8	4·1	—
	3(a)	4·3	4·3	—
	4(b)	7·4	3·6	—
	5(a)	7·5	7·2‡	—
	6(b)	9·2‡	9·9‡	—
	7	14·0‡	13·6‡	—
	8	16·2‡	12·4‡	—
Cord blood	1	2·9	4·5	0·8
	2	7·2	5·9	10·1
	3	7·6	6·4	8·6
	4	7·7	6·1	9·3
	5	7·8	11·2‡	15·9
	6	8·8‡	9·0‡	12·3
	7	13·3‡	11·5‡	13·2

Patients who had insufficient numbers of circulating B lymphocytes are indicated (—).
n.d. = Not determined.
* B cells were isolated by rosetting with anti-Ig OxRBC.
† Patients with childhood onset CV-H.
‡ Values within the normal adult range (mean ± 2 s.d.).
§ Patients who were brothers are indicated: (a), (b).

RESULTS

The circulating mononuclear cells of patients with X-H and CV-H and of cord blood had significantly less ($P < 0·001$) 5′-N activity than mononuclear cells isolated from normal adults (Fig. 1). Seven out of fourteen CV-H patients, five out of eight X-H patients, and eight out of eleven cord blood samples gave values below the normal adult range (mean ± 2 s.d.).

As shown in Table 1, eight of the thirteen CV-H patients studied showed a subnormal level of 5′-N on T cells. Sufficient numbers of B cells were obtained from seven patients: four of whom had a subnormal 5′-N activity on these cells. Three patients had a low 5′-N activity on both T and B cells, one patient showed a normal T cell value but subnormal B cell value, and another patient showed a normal B cell value with a low T cell value. All eight of the X-H patients had less than 1% circulating B lymphocytes, and 5 had a mononuclear cell 5′-N activity below the normal adult range. Four out of eight patients

TABLE 2. The effect of 1% triton X-100 detergent on the 5′-nucleotidase activity of circulating mononuclear cells

Source of cells	Mean activity of solubilized cells / activity of intact cells × 100 ± s.d.
Normal	136 ± 8 (n = 9)
Cord blood	141 ± 9 (n = 5)*
CV-H	147 ± 20 (n = 6)*
X-H	148 ± 23 (n = 4)*

* Not significant (Student's t-test, $P > 0{\cdot}1$).

TABLE 3. 5′-Nucleotidase activity (nmol AMP/hr/10^6 cells) of cells disrupted by freeze-thawing

Subjects	Intact cells	Freeze-thawed cells		
		Homogenate	Particulate* fraction	Soluble fraction
Normal	17·9	17·5	16·6	0·4
	13·3	12·3	12·5	0·3
	12·9	11·5	10·7	0·3
CV-H patients	17·9	18·5	17·7	0·4
	9·4	7·4	6·7	0·1
	1·7	1·1	0·6	0·0

* The 'particulate' fraction was obtained by centrifugation at 38,000 g for 45 min and washing with 10 mM tris-buffered H_2O, pH 7·5.

also revealed a low T cell 5′-N activity. Cord blood B lymphocytes had consistently less 5′-N than adult B cells, and four out of seven samples had a T cell 5′-N activity below the normal adult range.

When T and B cells are isolated from circulating mononuclear cells by positive selection, there is a residual population of cells (T^-sIg^-) which is predominantly monocytes. The T^-sIg^- cells from healthy adults and patients with CV-H had a 5′-N activity (nmol AMP/hr/10^6 cells) of 4·3 ± 2·5 (mean ± s.d., n = 8) and 5·6 ± 5·9 (n = 12) respectively. The low activity of T^-sIg^- cells may be considered an indication of the efficiency of T and B cell removal.

It is possible that some 5′-N activity may be masked because some of the enzyme is inside the cell, and therefore unavailable to extracellular substrate. Triton X-100 detergent increased the 5′-N activity of mononuclear cells from normals, patients, and cord blood to the same extent (Table 2). Freeze-thawing of mononuclear cells from CV-H patients did not increase the 5′-N activity (Table 3) and, as with normal cells, the bulk of the 5′-N activity was located in the 'particulate' fraction. Apparent Km values for the 5′-N on circulating mononuclear cells were similar for normal adults, cord blood, and hypogammaglobulinaemic patients (Fig. 2).

Circulating mononuclear cells from cord blood and patients with CV-H were cultured for 18 hr with an equal number of normal adult cells in order to investigate whether a soluble factor is produced which regulates the 5′-N activity. The enzyme activity of cell mixtures was always close to the calculated mean value of the two populations of cells cultured alone (Table 4). The results obtained with two controls show that short term culture of allogenic cells did not affect the 5′-N activity.

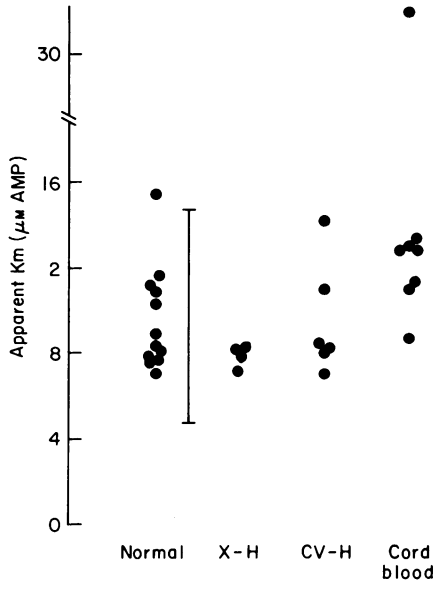

FIG. 2. Apparent Michaelis constant of 5′-nucleotidase (for AMP substrate) in healthy adults, patients with CV-H and X-H, and cord blood. The bar represents the normal adult range (mean ± 2 s.d.).

TABLE 4. 5′-Nucleotidase activities (nmol AMP/hr/10^6 cells) of circulating mononuclear cell mixtures maintained in culture for 18 hr

Source of cells	Experiment 1*	Experiment 2
Control A alone	15·6	16·1
Control B alone	10·6	19·1
Control A+Control B	13·9 (13·1)†	17·6 (17·6)
Patient alone	2·4	2·3
Patient+Control A	9·3 (9·0)	10·1 (9·2)
Patient+Control B	6·3 (6·5)	11·2 (10·7)
	Experiment 3	Experiment 4
Control C alone	14·7	13·6
Cord blood alone	4·7	3·8
Control+cord blood	7·9 (9·7)	8·1 (8·7)

* Each experiment was conducted with different controls and patients.

† Figures in parentheses denote the expected mean 5′-N activities of the cell mixtures.

DISCUSSION

Previous reports of low lymphocyte 5′-N activities in cord blood (Kramers *et al.*, 1977) and in patients with CV-H (Johnson *et al.*, 1977; Webster *et al.*, 1978) have been confirmed in this study. The group of X-H patients studied here had a wider range of 5′-N activity than previously reported; ranging from the low–normal values of Webster *et al.* (1978), to the very low values found by Edwards *et al.* (1978). We have shown that the low levels of 5′-N activity on the circulating mononuclear cells of many of these patients are due to a low T cell 5′-N activity in addition to the lack of circulating B cells. No consistent pattern of 5′-N activities was found in families of X-H patients. Low levels of mononuclear cell 5′-N

in CV-H patients result from one or more of three mechanisms: reduced T cell 5'-N activity, reduced B cell activity and a low percentage of B cells. However, the low level of 5'-N on T cells appears to be the predominant cause. Many CV-H patients have a normal percentage of circulating B cells (Cooper & Lawton, 1972) and may have a normal 5'-N activity on these cells. The loss of B cell 5'-N activity is not as consistent as their reported functional defect (Broom *et al.*, 1976; De La Concha *et al.*, 1977; de Gast *et al.*, in preparation). There is also no correlation between the level of 5'-N activity on T cells from CV-H patients and their ability to 'help' *in vitro* immunoglobulin production by normal B cells (de Gast *et al.*, in preparation).

That most cord blood samples had less 5'-N activity than healthy adults on their circulating mononuclear cells, appeared to be due to lower activities on both B and T lymphocytes. Also, although the proportion of B cells in cord blood is the same as in adult blood, there are fewer T cells and more monocytes (Kramers *et al.*, 1977; Berman & Johnson, 1978). Monocytes from cord blood and adult blood have relatively little 5'-N activity (Berman & Johnson, 1978; Rowe *et al.*, 1979).

Our results suggest that there is no abnormality of the enzyme itself in patients with hypogammaglobulinaemia. We have detected no inhibitor of 5'-N in patient lymphocytes, and the enzyme had a similar apparent Km to that of normal lymphocytes. We also found no evidence for abnormal compartmentalization of the enzymes. It therefore seems likely that there is either a reduced synthesis of the enzyme, or an absence of cells with a high 5'-N activity.

The 5'-N deficiency in patients with hypogammaglobulinaemia may reflect a stage of maturation arrest in T and B lymphocytes. Changes in 5'-N activity during cell development have been observed in human monocytes (Berman & Johnson, 1978; Rowe *et al.*, 1979); and other animal tissues. Chronic lymphatic leukaemia cells have a very low 5'-N activity (Quagliata *et al.*, 1974) and are thought to be arrested at an early stage of development (Salmon & Seligmann, 1974). Cord blood B and T cells may also be immature (Hayward & Lawton, 1977).

Our findings provide further evidence for a T cell abnormality in patients with CV-H and X-H. The B cells of CV-H patients sometimes had a normal level of 5'-N activity, indicating that there is no direct link between 5'-N activity and the synthesis of immunoglobulin by these cells. This view is supported by the recent observation that immunoglobulin production in normal lymphocytes was not affected by the presence of an inhibitor of 5'-N (Rowe & Johnson, 1979).

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