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Epstein–Barr-virus-transformed lymphoblastoid cell lines derived from patients with X-linked agammaglobulinaemia and Wiskott–Aldrich syndrome: responses to B cell growth and differentiation factors

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SUMMARY

Epstein–Barr-virus-transformed B lymphoblastoid cell lines (EBV-transformed LCL) from three patients with X-linked agammaglobulinaemia (XLA), six patients with Wiskott–Aldrich Syndrome (WAS), and seven normal donors, were tested for growth and differentiation in response to human recombinant IL-4, a commercially available, low molecular weight B cell growth factor ($BCGF_{low}$), and B cell differentiation factor (BCDF) secreted by the T24 cell line, now known to be IL-6. Proliferation (^{3}H-TdR uptake) by EBV-transformed LCL from both XLA and WAS patients in response to $BCGF_{low}$ was similar to that obtained with the normal cell lines. In addition, three normal and three WAS, but none of the XLA EBV-transformed LCL, proliferated a little in response to IL-4. All the normal B cell lines secreted IgM, and six out of the seven secreted IgG in response to $BCGF_{low}$ and BCDF. A similar pattern of response was obtained with the WAS EBV-transformed LCL (6/6 secreted IgM and 4/6 secreted IgG). Several of the normal and WAS EBV-transformed LCL also secreted IgM and IgG in response to IL-4. In contrast, the lines from the XLA patients were abnormal. One secreted large amounts of IgM and two secreted small amounts, but none of the XLA lines secreted IgG constitutively or in response to any of the factors (IL-4, $BCGF_{low}$, BCDF). The lack of detectable IgG secretion by the XLA lines was probably due to an absence of precommitted IgG B cell precursors transformed by EBV rather than an intrinsic inability to respond to BCGF and BCDF. All of the lines, including those derived from XLA patients, were shown to secrete B cell growth and differentiation factors detected on indicator B cell lines. These results suggest that the abnormal X-linked genes responsible for XLA and WAS do not interfere with B cell responses to B cell growth and differentiation factors.

Keywords B cell lines growth factor differentiation factor EBV X-linked immunodeficiency agammaglobulinaemia Wiskott–Aldrich

INTRODUCTION

At least eight inherited immunodeficiency diseases are linked to the X-chromosome (Lau & Levinsky, 1988). Of these, both X-linked agammaglobulinaemia (XLA) and Wiskott–Aldrich Syndrome (WAS) have been shown by both immunological and genetic studies to involve the B cell lineage. In patients with XLA normal numbers of pre-B cells are present in bone marrow, but very few B cells are found in blood or lymphoid tissues (Siegal, Pernis & Kunkel, 1971; Pearl *et al.*, 1978; Conley, 1985). Peripheral blood mononuclear cells (PBMC) from XLA patients do not make antibody in response to pokeweed mitogen (PWM) (Choi, Biggar & Good, 1972; Herrod & Buckley, 1979), but detectable levels of serum immunoglobulins are sometimes found, and IgM-secreting, Epstein–Barr-virus-transformed (EBV-transformed) B cell lines have been obtained from blood and bone marrow cells (Fu *et al.*, 1980; Levitt, Ochs & Wedgewood, 1984). In some EBV-transformed LCL from XLA patients, complete V_HDJ_H rearrangements are known to occur (Mensink *et al.*, 1986), but truncated C-mu with no V_H segment in pre-B cells from XLA patients and obligate carriers have also been described (Schwaber *et al.*, 1983). More recently, non-random X-chromosome inactivation in B cells from obligate female carriers of XLA has been reported (Fearon, 1987) showing that B cells expressing the genetic defect do not mature in a normal host. Similar findings have been reported in patients with WAS. An undefined B cell defect has been assumed from the inability of these patients to respond normally to T cell independent polysaccharide antigens (Blaese *et al.*, 1968),

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although B cells from WAS patients can respond to a T independent type 1 antigen (TNP-*Brucella abortis*) (Golding, Muchmore & Blaese, 1984). Non-random X-chromosome inactivation occurs in both T cells and B cells in obligate carriers of WAS, suggesting that the defect affects cells of both lineages (Prchal *et al.*, 1980; Kohn *et al.*, 1987; Blaese, *personal communication*).

It is clear from these considerations that the primary genetic abnormalities in both XLA and WAS have a direct effect on cells of the B cell lineage, but the cell functions controlled by these genes are unknown. One possibility is that affected B cells are unable to secrete or respond to B cell growth and differentiation factors (BCGF and BCDF), but very little is known of the way B cells from patients with XLA and WAS respond to BCGF and BCDF due to the difficulty of obtaining sufficient numbers of B cells for investigation, especially from patients with XLA. Recent work has shown that EBV-transformed lymphoblastoid cell lines (LCL) both secrete, and respond to, several factors controlling B cell growth and differentiation (Gordon, Guy & Walker, 1985; Hirano *et al.*, 1986; Muraguchi *et al.*, 1986; Swendeman & Thorley-Lawson, 1987). In the present study, we prepared EBV-transformed LCL from XLA and WAS patients (which must carry the defective gene on the X-chromosome) and tested these lines for production of, and response to, BCGF and BCDF.

MATERIALS AND METHODS

Patients

All three XLA patients had low levels of IgG, IgA and IgM, and low numbers of recirculating B cells. The pedigree of patient RH was originally reported by Jamieson & Kerr (1962) and the EBV-transformed LCL from this patient was obtained from Dr Roifman. A second EBV-transformed line (MH) was obtained from Professor R. Buckley, and the family of this patient described by Leickley & Buckley (1986). The third patient (BW) was under our care and the family has been described as part of a larger study with linked DNA probes (Lau *et al.*, 1988). Six patients, all from different families, were diagnosed as having WAS either in the Hospital for Sick Children, London (GB, DR, SS, MC), or Booth Hall Hospital, Manchester (RF, PM). They all had the characteristic profile of WAS (eczema, thrombocytopaenia, low levels of isohaemagglutinins, and variable degrees of T cell deficiency) (Rosen *et al.*, 1983).

Cell Lines

Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood by centrifugation over Ficoll-Hypaque (1·077 kg/l). EBV-transformed LCL were obtained by incubating between 5×10^6–20×10^6 PBMC with 1 ml of supernatant from the EBV-secreting marmoset cell line B958 for 1 h at 37°C. Infected cells were then washed and cultured at 1×10^6 cells/ml in RPMI 1640 containing 25 mM HEPES, 5% fetal calf serum (FCS, Gibco, Paisley, GB) 2 mM L-glutamine and 1 μg/ml of cyclosporin A in 24-well culture plates (Linbro, Flow Laboratories). Clumps of cells were usually seen at the end of 2 weeks, and stable EBV lines established by the end of 4 weeks. A total of seven normal, six WAS and one XLA B cell lines were obtained in this way. Two other EBV-transformed LCL from XLA patients were obtained from Professor R. Buckley and Dr C. Roifman. All EBV-transformed LCL established in our laboratory were studied within 2 months of being established. The indicator B cell lines (L4, HFB-1 and CESS) were used to assay growth and differentiation factors secreted by EBV-transformed LCL as described elsewhere (Muraguchi *et al.*, 1981; Shields *et al.*, 1987).

Table 1. Proliferation of EBV-transformed LCL in response to $BCGF_{low}$ and IL-4

EBV-transformed LCL		Proliferation (^{3}H-TdR dpm)		
		Medium	$BCGF_{low}$	IL-4
Normal	SH	23 507 ± 2 600	109 317 ± 9 381	21 055 ± 885
	VR	5 448 ± 524	28 944 ± 9 780	6 051 ± 1 033
	JS	5 165 ± 983	18 941 ± 2 663	6 556 ± 692
	EG	3 062 ± 526	60 613 ± 5 629	16 337 ± 3 184*
	BJ	19 049 ± 2 588	134 514 ± 7 375	30 267 ± 3 184*
	JR	19 933 ± 889	68 807 ± 9 841	45 824 ± 4 331*
	DB	30 065 ± 7 866	123 651 ± 31 859	24 483 ± 1 132
XLA	BW	9 806 ± 3 185	27 966 ± 11 965	7 549 ± 1 632
	RH	6 125 ± 472	249 266 ± 19 42	9 359 ± 1 867
	MH	12 864 ± 2 149	75 812 ± 6 056	2 392 ± 374
WAS	GB	13 716 ± 1 115	208 112 ± 61 777	29 317 ± 1 818*
	DR	44 702 ± 6 046	92 477 ± 7 749	34 832 ± 1 856
	PM	38 268 ± 694	236 951 ± 4 882	34 213 ± 2 677
	RF	15 982 ± 1 358	208 549 ± 10 733	31 294 ± 2 752*
	SS	7 528 ± 1 327	25 280 ± 10 707	17 742 ± 5 015*
	MC	22 256 ± 3 405	97 710 ± 5 535	32 809 ± 7 172

* significant proliferation in response to IL-4.

Sources of factors

Purified low molecular weight BCGF ($BCGF_{low}$), prepared by Cellular Products, was purchased from Sera-Lab (Crawley Down, GB). This factor was originally described by Maizel (Maizel *et al.*, 1982; Mehta *et al.*, 1985). Human BCDF (IL-6/BSF2)-containing supernatants were obtained from the bladder carcinoma line T24 (Rawle *et al.*, 1986). The T24-BCDF secreted by this line is indistinguishable from IL-6 (BSF-2), as reported by Hirano *et al.*, (1986). Supernatants from COS-7 cells transfected with plasmids bearing the human IL-4 gene, supplied by Dr J. Banchereau, Unicet, France, were used as a source of recombinant human IL-4 (rhIL-4) (Yokota *et al.*, 1986). BCGF- and BCDF-containing supernatants from normal and XLA EBV-transformed LCL were filtered through 0·2 μm Acrodisc filters (Gelman Sciences, GB) and stored at −70°C before use.

Responses of EBV-transformed cell lines and indicator B cell lines to BCGF and BCDF

Cell lines were sub-cultured 24–48 h before use in order to ensure log-phase growth. The cells were then harvested, washed and cultured in HEPES-buffered RPMI 1640 supplemented with 2 mM glutamine and FCS (2% in proliferation assays and 5% in differentiation assays) at four different concentrations ranging between 1 and 20×10^3 cells/well in 96-well flat-bottomed microtitre trays. Maximum responses were always obtained from within this range, but the optimal cell concentration required did vary from experiment to experiment (Shields *et al.*, 1987). Factors were added to give final concentrations of 10% ($BCGF_{low}$), 20% (T24-BCDF and supernatants from EBV

Table 2. IgG secretion by EBV-transformed LCL

		IgG (ng/ml)			
		Medium	$BCGF_{low}$	T24-BCDF	IL-4
Normal	SH	441±45	1430±420	2031±516	678±180
	VR	249±33	1439±399	1539±420	183±12
	JS	6±3	513±72	69±12	15±3
	EG	24±3	581±174	273±57	69±48
	BJ	9±3	69±15	57±21	30±6
	JR	57±12	459±108	477±18	153±39
	DB	<3	<3	<3	<3
XLA	BW	<3	<3	<3	<3
	RH	<3	<3	<3	<3
	MH	<3	<3	<3	<3
WAS	GB	81±3	1215±567	336±123	396±96
	DR	<3	<3	<3	<3
	PM	87±30	>3000	>3000	102±9
	RF	27±9	408±230	426±45	54±39
	SS	912±174	1635±591	2337±606	984±141
	MC	<9	<9	<9	<9

Table 3. IgM production by EBV-transformed LCL

		IgM (μg/ml)			
		Medium	$BCGF_{low}$	T24-BCDF	IL-4
Normal	SH	<0·06	1·7±0·1	0·8±0·2	ND
	VR	0·9±0·2	8·1±0·2	11·3±0·0	0·5±0·4
	JS	0·8±0·0	24·0±4·5	8·1±0·8	2·0±0·3
	EG	0·5±0·2	11·8±1·4	9·5±1·4	0·6±0·2
	BJ	1·3±0·2	9·9±0·8	5·8±1·4	3·1±0·5
	JR	1·2±0·5	16·8±8·9	6·7±3·9	8·1±3·3
	DB	0·5±0·1	18·8±2·7	9·8±1·5	0·5±0·1
XLA	BW	0·5±0·2	4·8±1·2	1·8±0·4	0·3±0·1
	RH	1·3±0·2	36·5±3·0	16·8±1·4	0·5±0·1
	MH	0·7±0·2	4·2±0·8	4·0±0·4	1·2±0·1
WAS	GB	17·4±4·4	94·5±26·8	62·7±10·4	35·3±7·7
	DR	8·0±0·7	21·0±6·1	16·8±2·7	8·1±1·1
	PM	0·4±0·2	3·5±0·3	4·1±0·6	0·9±0·6
	RF	0·3±0·0	24·3±0·6	9·9±3·8	0·5±0·2
	SS	0·5±0·4	3·2±1·2	8·9±4·2	2·9±1·1
	MC	6·8±0·7	28·5±9·6	49·7±4·1	72·7±47·3

ND not done.

transformed LCL), and 1% (rhIL-4, approximately 500 units/ml). When assaying for proliferation, cultures were incubated at 37 °C for 3 days and pulsed with 1 μCi/well (37 kBq) of ^{3}H-TdR for the last 8 h. Results are given as the mean dpm of incorporated ^{3}H-TdR from triplicate cultures (±1s.d.). When assaying for differentiation activity, the cultures were incubated for 5 days and immunoglobulin levels in the supernatant measured by enzyme-linked immunosorbent assay (ELISA) (Shields & Turner, 1986). Results are given as the mean ± 1s.d. of triplicate cultures. Responses of indicator B cell lines to BCGF and BCDF secreted by EBV-LCL were determined in the same way.

Table 4. B cell growth factor secreted by normal and XLA EBV-transformed LCL

	Proliferation (^{3}H-TdR dpm)	
Source of BCGF	L4	HFB1
Medium	4482±230	6748±2047
BCGF (10%)	148698±10044	74343±16103
Normal EBV-LCL	55981±3726	150622±4198
XLA EBV-LCL	199348±7378	179882±8559

Table 5. BCDF production by normal and XLA EBV-transformed LCL

	IgG (ng/ml)	
	L4	CESS
Medium	<9	39±18
T24	180±21	966±129
Normal EBV-LCL	118±21	507±141
XLA EBV-LCL	258±6	216±45

RESULTS

Proliferative response of EBV-transformed LCL to $BCGF_{low}$ and IL-4

EBV-transformed cell lines from seven normal subjects, three XLA and six WAS patients were tested for their proliferative responses to $BCGF_{low}$ and IL-4. In these experiments the starting cell concentration was found to be critical. At high cell concentrations the effect of growth factors was minimal, while at very low cell concentrations little proliferation occurred even in the presence of growth factors (Callard, Shields & Smith, 1987). The results obtained with the optimal cell concentration for each cell line are presented in Table 1. All the normal, XLA and WAS EBV-transformed LCL responded significantly to $BCGF_{low}$. In addition, three normal (EG, BJ and JR), and three WAS (GB, RF and SS), lines responded to some extent with rhIL-4. None of the three XLA lines proliferated in response to rhIL-4.

Differentiation of EBV-transformed LCL in response to $BCGF_{low}$, T24-BCDF and IL-4

The EBV-transformed LCL shown in Table 1 were also tested for differentiation (increased IgG and IgM secretion) in response to $BCGF_{low}$, T24-BCDF and IL-4. The results obtained with optimal cell concentrations for each cell line are presented in Tables 2 and 3. All normal EBV-transformed LCL increased IgM secretion in response to $BCGF_{low}$ and T24-BCDF, and all but one secreted IgG. Unexpectedly, some normal EBV-transformed LCL (BJ, JS, JR) also increased both IgG and IgM production in response to rhIL-4. The EBV-transformed LCL from WAS patients responded in much the same way. All six lines secreted IgM in response to $BCGF_{low}$ and T24-BCDF, and all but two increased IgG production. Three of these lines (GB,

SS, MC) also increased IgG and/or IgM secretion in response to IL-4.

In contrast, the responses of EBV-transformed LCL from XLA patients to BCDF were abnormal. No IgG secretion could be detected either constitutively, or in response to $BCGF_{low}$ or T24-BCDF. Two of the three (BW and MH) produced slightly more IgM in response to $BCGF_{low}$ and T24-BCDF, whereas the third line (KH) significantly increased IgM production. None of the XLA EBV-LCL increased Ig secretion in response to IL-4.

B cell growth and differentiation factors secreted by normal and XLA EBV-transformed LCL

B cell growth and differentiation factors present in supernatants of normal and XLA EBV-transformed LCL were assayed using the indicator lines L4, HFB-1, and CESS (Tables 4 and 5). Supernatants from both normal and XLA EBV-transformed LCL contained BCGF detected with L4 and HFB-1. In each case the response was comparable to that obtained in control cultures with $BCGF_{low}$. Both normal and XLA EBV-transformed LCL also secreted BCDF detected with L4 and CESS (Table 5).

DISCUSSION

EBV-transformed LCL secrete and respond to several distinct B cell growth and differentiation factors. These include a B cell derived IL-1 (Matsushima *et al.*, 1985; Gordon, Guy & Walker 1986; Rimsky *et al.*, 1986), high and low molecular weight BCGF (Ambrus *et al.*, 1985; Muraguchi *et al.*, 1986; Buck *et al.*, 1987), an Mr25K soluble fragment derived from CD23 (s-CD23) (Swendeman & Thorley-Lawson, 1987; Cairns *et al.*, 1988), and IL-6 (BSF-2) (Hirano *et al.*, 1986), but not usually IL-2 or IL-4. Such lines may not be totally representative of normal B cell populations (Chan *et al.*, 1986), but their range of responses to B cell growth and differentiation factors, and availability in large numbers free of other cell types makes them particularly suitable for studies of some aspects of B cell physiology. In the present study EBV-transformed LCL were obtained from WAS and XLA patients. Particularly in the latter, it is extremely difficult to obtain sufficient numbers of recirculating B cells for study, and the use of LCL from these patients is the only reasonable option for measuring responses to BCGF and BCDF. In both WAS and XLA, the inherited defect is known, from both functional and genetic studies, to be expressed in cells of the B cell lineage (Blaese *et al.*, 1968; Prchal *et al.*, 1980; Schwaber *et al.*, 1983; Golding *et al.*, 1984; Fearon *et al.*, 1987; Kohn *et al.*, 1987; Blaese, *personal communication*). If the genetic defect in these immunodeficiencies was phenotypically expressed in B cells by an inability to secrete or respond to B cell growth or differentiation factors, this should be detectable by transformed B cells or normal B cells. The EBV-transformed LCL used in this study were grown for a short period only (8–12 weeks), in bulk cultures to avoid overgrowth by dominant clones. In every case tested, cell lines grown in these conditions were polyclonal as indicated by secretion of more than one immunoglobulin isotype (IgG, IgM and IgA) (Shields *et al.* in preparation).

EBV-transformed LCL from both XLA and WAS patients proliferated in response to $BCGF_{low}$ (Table 1). In each case the response was not appreciably different from that of normal EBV-transformed LCL. Similar results were obtained with PHA-conditioned medium (data not given). Unexpectedly, rhIL-4 (BSF-1) promoted cell division in some normal and WAS EBV-transformed LCL, although it had no effect on the XLA EBV-transformed LCL (Table 1). IL-4 is a BCGF in co-stimulation assay with anti-IgM for normal B cells (Defrance *et al.*, 1987) but does not usually induce proliferation of B cell lines (Shields *et al.*, 1987). The reason why some normal and WAS EBV-transformed LCL proliferate in response to IL-4 is unclear. It may be a property of B cell subpopulations infected with EBV, or of B cells at a particular stage of differentiation or activation represented by these lines.

In the differentiation assays both normal and WAS EBV-transformed LCL increased secretion of IgM and IgG in response to $BCGF_{low}$ and to T24-BCDF (Tables 2 and 3). $BCGF_{low}$ is a purified factor from activated T cells which is known to increase Ig secretion by B cell lines (Shields *et al.*, 1987). The T24 bladder carcinoma line is known to secrete IL-6 (BSF-2) (Hirano *et al.*, 1986; Rawle *et al.*, 1986) which is a BCDF for EBV-transformed LCL (Hirano *et al.*, 1986). Interestingly, some normal and WAS EBV-transformed LCL also increased IgM and IgG secretion in response to IL-4. This is consistent with our observation that rhIL-4 is a differentiation factor for freshly prepared EBV-transformed LCL (Shields *et al.*, in preparation). EBV-transformed LCL obtained from XLA patients did not respond normally in the differentiation assays (Tables 2 and 3). Two of the three lines made small amounts of IgM, and the third line made large amounts of IgM in response to $BCGF_{low}$ and to T24-BCDF. This is consistent with earlier findings in which unstimulated EBV-transformed LCL from patients with XLA were shown to secrete IgM (Levitt *et al.*, 1984). No detectable IgG was secreted by any of the lines derived from XLA patients in the presence or absence of B cell differentiation factors, and none of the XLA EBV-transformed LCL responded to IL-4.

Patients with XLA do have low levels of serum IgG. The lack of IgG production by the XLA-transformed LCL may, therefore, have been due to an inability to transform pre-committed IgG precursors with EBV (Stein Ledgley & Sigal, 1983; Chan *et al.*, 1986), or a failure to switch from IgM to IgG. Various combinations of factors (IL-4 and $BCGF_{low}$; IL-4 and T24-BCDF; or $BCGF_{low}$ and T24-BCDF) were tried in an attempt to induce switching of the IgM-producing EBV-transformed LCL from the XLA patients and the one normal and two WAS EBV-transformed LCL which did not secrete detectable IgG, but without success (data not presented). We are currently investigating this question by limiting dilution analysis of IgG, IgM and IgA precursor frequencies.

B cells are known to secrete, as well as respond to, growth and differentiation factors (Gordon, Guy & Walker, 1985; Muraguchi *et al.*, 1986). For example, EBV-transformed LCL depend on an autocrine growth factor for continuous proliferation (Gordon *et al.*, 1984), and normal B cells may also depend on autostimulation to allow rapid expansion of proliferating clones in areas of high B cell density. It was, therefore, of interest to determine whether B cells derived from our patients were able to produce autocrine factors. Using the indicator B cell lines for assaying B cell derived growth and differentiation factors,

supernatants from XLA EBV-transformed LCL were shown to contain BCGF and BCDF (Tables 4 and 5). A number of different factors have been shown to be important for autocrine proliferation by EBV-transformed LCL (Ambrus *et al.*, 1985; Matsushima *et al.*, 1985; Gordon, Guy & Walker, 1986; Muraguchi *et al.*, 1986; Buck *et al.*, 1987; Swendeman & Thorley-Lawson, 1987; Cairns *et al.*, 1988), but which of these factors were secreted by the XLA EBV-transformed LCL was not investigated further.

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