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Autocrine models of B-lymphocyte growth

II. INTERLEUKIN-1 SUPPORTS THE PROLIFERATION OF TRANSFORMED LYMPHOBLASTS BUT NOT THE STIMULATION OF RESTING B CELLS TRIGGERED THROUGH THEIR RECEPTORS FOR ANTIGEN

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SUMMARY

Purified, monocyte-derived interleukin-1 (IL-1) was found to provide growth support for Epstein-Barr virus (EBV) transformed B-lymphocytes seeded at densities below which their own autostimulatory factors were limiting. By contrast, highly purified resting B cells triggered via their receptors for antigen failed to respond to identical preparations of IL-1 by DNA synthesis. That successful priming of the B cells had occurred was evidenced by a transient rise in RNA synthesis and the ability of the cells to respond to T-cell supernatants by DNA synthesis. The findings indicate that while IL-1 might perform an autostimulatory function in B lymphocyte proliferation it is not by itself sufficient to provide growth support for resting B cells activated through their receptors for antigen. The implications of these observations for autocrine models of B-cell growth are discussed.

INTRODUCTION

Following low level triggering through their receptors for antigen, B lymphocytes are primed to respond to a variety of soluble growth mediators of monocyte and T-cell origin (Howard & Paul, 1983). There does exist, however, certain classes of activators which drive resting B cells to DNA synthesis without the apparent intervention of contaminating cells or factors derived from them (Falkoff *et al.*, 1983). In a previous paper we have shown that for both the non-transforming activator SAC (*Staphylococcus aureus* Cowan Strain I) and the transforming virus EBV this is achieved, at least in part, through an apparent autocrine mechanism (Gordon, Guy & Walker, 1985). Such a mechanism is exemplified by EBV-transformed cell lines which emerge through a maintenance of what may be a normally transient autocrine phenotype (Blazar *et al.*, 1983; Gordon *et al.*, 1984a).

The precise nature of the molecules involved in B-cell growth and the details of their action are not yet clear. Probably the best characterized of the molecules implicated is the T-cell derived protein termed B-cell stimulatory factor 1 (BSF-1). This lymphokine acts on resting B lymphocytes to prepare them for S phase entry on subsequent exposure to low levels of anti-immunoglobulin (Rabin, O'Hara & Paul, 1985). Two other T-cell products, interleukin-2 (IL-2) (Nakagawa *et al.*, 1985) and BCGFII (Yoshizaki *et al.*, 1983) appear to provide a proliferation signal for B cells which have already been activated in appropriate modes. Apart from the T-cell-derived molecules, the monocyte product interleukin-1 has also been implicated in B-cell growth (Howard *et al.*, 1983).

The exact role of interleukin-1 (IL-1) in B-cell growth responses remains controversial. Some claim it acts as an essential co-factor with BSF-1 for driving anti-immunoglobulin primed cells into DNA synthesis (Howard *et al.*, 1983). Others suggest that it can offer a modest growth stimulus by itself to B lymphocytes excited through their receptors for antigen (Falkoff *et al.*, 1983). Booth & Watson (1984) have recently suggested that IL-1 also acts on B cells primed in a mode conventionally designed to demonstrate BCGFII activity. These workers consider that IL-1 alone is sufficient to provide the signal for entry into DNA synthesis for both anti-immunoglobulin and dextran sulphate stimulated B cell subsets.

With regard to autocrine models of B lymphocyte growth the observations on IL-1 are particularly intriguing as the production of this activity has been shown to be associated with cells of the B lineage. Both normal (Rock, Benecarraf & Abbas, 1984) and transformed B cells (Issekutz, Chu & Geha, 1982) are capable of providing accessory function in T-cell responses and molecules with IL-1 activity have been found in the supernatants of EBV-transformed lymphoblastoid lines (Scala *et al.*, 1984).

In this paper we describe experiments which investigate whether purified IL-1 can substitute for autocrine factors in the growth of EBV-transformed lymphoblasts. Furthermore, we attempt to clarify the role of IL-1 in the response of resting B cells to anti-immunoglobulin by using populations which have

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been higly purified with respect to contaminating T cells and monocytes.

MATERIALS AND METHODS

Cell lines

Lymphoblastoid cell lines (LCL) were derived from normal healthy individuals as described previously (Gordon *et al.*, 1984c). Cultures were carried in RPMI-1640 supplemented with 10% fetal calf serum, L-glutamine (mM), 2-mercaptoethanol (5×10^{-5} M) and antibiotics. This constituted the full medium used throughout these studies. Conditioned medium was collected as described previously (Gordon *et al.*, 1984a).

B-cell preparation

B cells were isolated from tonsillar tissue by negative selection as described in detail elsewhere (Gordon, Guy & Walker, 1985). The high density fraction passing through a Percoll (Pharmacia, Uppsala, Sweden) gradient adjusted to 57·5% provided the 'resting' B cells used in this study. The typical profile of populations thus prepared has been detailed (Gordon, Guy & Walker, 1985). To reiterate: no cell among 500 scored stained for non-specific esterase or reacted with pan-monocyte monoclonal antibodies; no cell among 500 scored formed rosettes with AET-modified sheep erythrocytes or reacted with pan-T-cell monoclonal antibodies; >98% of cells were surface immunoglobulin positive and >99% reacted with pan-B-cell antibodies. Only preparations which fulfilled these criteria were accepted into this study.

Cell cultures

Resting B cells were cultured at 10^5 per well in flat-bottom 96-well Nunclon (Nunclon, Kamstrup, Denmark) tissue culture plates in a final volume of 200 μl unless otherwise indicated. LCL cells were taken from culture, washed four times and seeded in fresh full medium at numbers indicated in a final volume of 200 μl in flat-bottomed microwells. $F(ab')_2$ preparations of goat IgG antibody to human μ-chains (Cappel, Cochranville, PA) were included in cultures as indicated at concentrations of 15–100 μg/ml. *Staphylococcus aureus* Cowan Strain I (SAC) (Calbiochem, La Jolla, CA) was included where indicated at 1:20 000. Affinity-purified interleukin-1 was purchased from Genzyme (Boston, MA) and constituted the 15K product of stimulated macrophages. On arrival, samples were aliquoted, stored at −70° and thawed only once before use at concentrations indicated in the text. T-cell conditioned medium was generated by a 48 hr exposure of E-rossette positive tonsillar cells to phytohaemagglutinin (Gibco, Paisley, Renfrewshire) 1:100 and collecting the supernatant from washed cells suspended in lectin-free medium for a further 24 hr. Conditioned medium was included in cultures at 10% where indicated.

Measurement of B-cell activation

RNA synthesis was assessed at 24, 48 and 72 hr by pulsing wells with 0·5 μCi of [^{3}H]uridine (UdR) for the final 16 hr of culture. DNA synthesis was similarly assessed at 72 hr by pulsing wells with 0·5 μCi of [^{3}H]thymidine (TdR). Cultures were then harvested and incorporated radioactivity counted. All determinations were performed in triplicate with mean values given. Individual determinations were usually within 5% of each other and never varied by greater than 10%.

Interleukin-1 assay

Activity of IL-1 preparations was assessed by the thymocyte comitogenesis assay as described by Mizel, Oppenheim & Rosenstreich (1978). Briefly, thymocytes from young mice (6–10

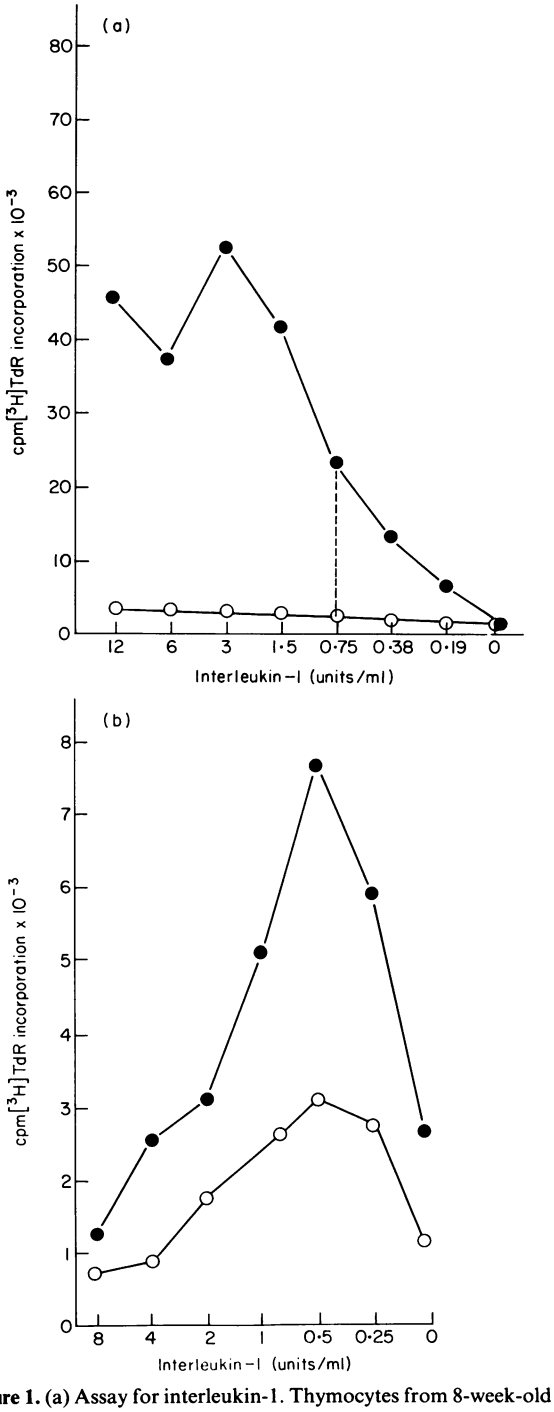

Figure 1. (a) Assay for interleukin-1. Thymocytes from 8-week-old mice were cultured in the presence (●) or absence (○) of PHA (1:1000) and indicated amounts of IL-1 as stated by the manufacturers. DNA synthesis at Day 3 was assessed by pulsing wells with [^{3}H]TdR for the final 16 hr. (b). Effect of interleukin-1 on LCL proliferation. Cells from two EBV-transformed LCL (○, ●) were seeded at 2000 per well in a volume of 200 μl with indicated amounts of IL-1. DNA synthesis at Day 3 was assessed as above.

weeks) were incubated with a sub-mitogenic dose of phytohaemagglutinin (PHA) (1:1000) at 10^6 per flat-bottom microwell in a final volume of 200 μl. The incorporation of [^{3}H]TdR at 72 hr in the presence of dilutions of IL-1 was assessed as described for B-cell cultures.

RESULTS

Capacity of IL-1 to provide growth support for EBV-transformed cells

For all experiments described in this paper, the preparations of IL-1 used against transformed or normal B cells were tested in parallel in a standard thymocyte co-stimulation assay designed to measure IL-1 activities. This was deemed necessary due to the known lability of preparations containing IL-1.

As shown in Fig. 1a, the affinity purified IL-1 yielded a 50% maximum response in the thymocyte co-stimulation assay at the manufacturer's stated value of 0·8 units/ml. In all subsequent IL-1 assays 50% maximum stimulation was achieved at or around this figure. The IL-1 preparations displayed some direct stimulatory activity for thymocytes in the absence of mitogens although this was low by comparison with the direct mitogenic response.

When cells from two EBV-transformed LCL were seeded at low density, the addition of IL-1 enhanced their proliferation in a dose-dependent manner as shown in Fig. 1b. Optimal growth enhancement was achieved at 0·5 u/ml of IL-1. Preparations containing greater than 0·5 u/ml yielded less than optimal growth support while above 2 u/ml, the preparations were actively inhibiting growth. The nature of this inhibitory effect is presently unknown but it is also found when using autologous supernatants as the source of growth-stimulating activity for lymphoblastoid lines (Blazar, Sutton & Strome, 1983). The results depicted in Fig. 2 show the influences of IL-1 at its optimal concentration (0·5 u/ml) on the growth of LCL cells seeded at varying numbers in culture. It can be seen that IL-1 provided similar, but not quite as potent, growth support for the transformed cells when compared with conditioned medium harvested from the cell line.

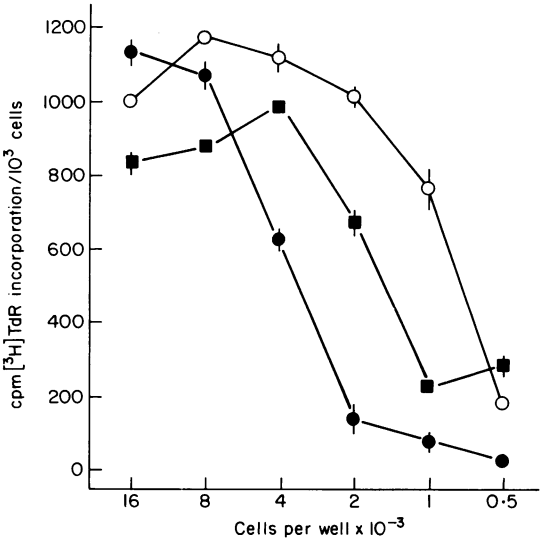

Figure 2. Influence of interleukin-1 on LCL cells of varying densities. Cells from a LCL (●) seeded at varying numbers in the presence (■) or absence (●) of IL-1 at 0·5 units/ml. DNA synthesis at Day 3 was assessed as described in Fig. 1. The influence of medium conditioned by autologous cells (final concentration 25%) on LCL proliferation is shown for comparison (○). Error bars with standard deviations are indicated.

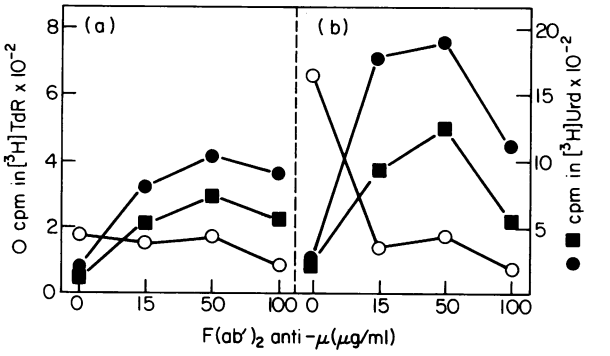

Figure 3. Excitation of purified resting B cells by anti-μ. Resting B cells from two donors (a, b) were incubated at 10^5 per well in a final volume of 200 μl for 0–72 hr with indicated concentration of F(ab')$_2$ anti-μ. RNA synthesis at 24 hr (●) and 48 hr (■) and DNA synthesis at 72 hr (○) are shown.

Activation of resting B cells by anti-immunoglobulin

The resting B-cell preparations used in this study were selected for their high degree of purity by the criteria outlined in 'Materials and Methods'. Only those preparations where cells with T-lymphocyte or monocyte features were absent among 500 scored were included. Furthermore, the B-cell preparations comprised lymphocytes with a G_0 staining pattern as revealed by acridine orange and FACS analysis. It was therefore necessary to show that such highly-enriched populations were capable of being activated by soluble anti-immunoglobulin.

Resting B cells were routinely plated at 10^5 per well in the presence of varying concentrations of soluble F(ab')$_2$ anti-μ. Under these conditions, using purified preparations, no DNA

Table 1. Response of resting B cells to T-cell supernatants

Anti-μ (μg/ml)	[^{3}H]TdR uptake (c.p.m.) in presence of: Control	T-CM*
0	374	621
15	286	14 308
50	449	17 655
100	162	8076

* T-cell conditioned medium added to 10% in cultures of resting B cells (10^5 per well in 200 μl) for 72 hr and pulsed with [^{3}H]TdR for final 16 hr.

synthesis above background was measured with any concentration of anti-μ used. Indeed, anti-μ tended to suppress the small amount of basal DNA synthesis already occurring. Representative results of the DNA synthesis occurring at Day 3 of exposure to anti-μ is shown for two preparations in Fig. 3. The cells used were clearly capable of mounting a good DNA response to a strong 'direct' stimulus via their antigen receptors as evidenced by their [^{3}H]TdR uptake in the presence of SAC: 23,407 c.p.m. for preparation 'a'; 43,920 c.p.m. for preparation 'b'.

Two independent lines of evidence demonstrated that the resting B cells were excited on exposure to soluble anti-μ. Firstly, significant RNA synthesis was induced during the first 24 hr of culture when anti-μ was present at 15–100 μg/ml (Fig. 3). This began to decline by Day 2 and was virtually absent on Day 3 (not shown). Secondly, cells exposed to anti-μ mounted a clear DNA response in the presence of crude T-cell conditioned medium which was not seen in the absence of the ligand (Table 1).

Influence of IL-1 on the response of resting B cells to anti-immunoglobulin

The same IL-1 preparations which were active in the thymocyte co-stimulation assay and provided growth support for transformed lymphoblasts were assessed for their ability to provide a growth signal for resting B cells primed by soluble anti-μ.

The results shown in Fig. 4 demonstrate conclusively that between the range of 0·1 and 4 u/ml, IL-1 was incapable of providing any support for a DNA response in purified B cells excited through their receptors for antigen. In other experiments

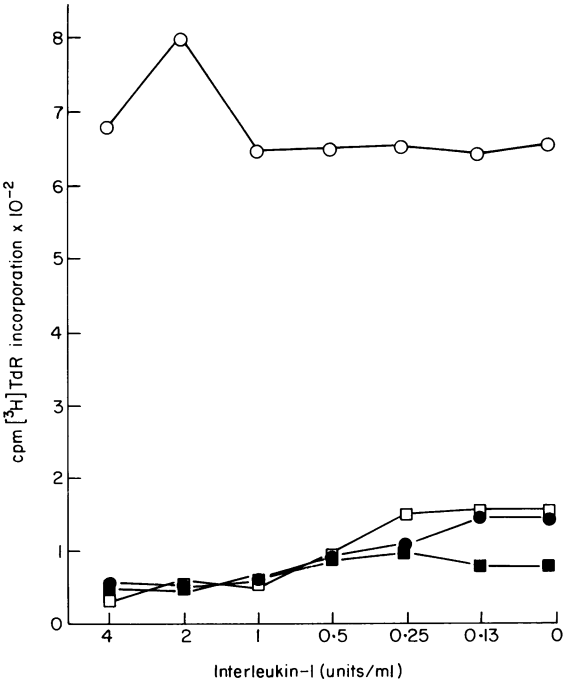

Figure 4. Influence of interleukin-1 on excited B cells. Cells from donor 'b' were cultured with IL-1 at indicated concentrations in the presence of anti-μ at 15 (□), 50 (●), 100 (■) μg/ml or in the absence of antibody (○). DNA synthesis at 72 hr is shown. The same experiments performed with three separate B-cell preparations yielded qualitatively identical results.

(not shown) it was found that increasing amounts of IL-1 (up to 20 u/ml) were also ineffective. Doubling the concentration of B cells (to 2×10^5 per well) or performing the experiments in round-bottom as opposed to flat-bottom wells did not alter the outcome of these findings (data not shown). It should be stressed that the populations shown not to respond to IL-1 invariably mounted a proliferative response to T-cell conditioned medium as indicated in Table 1. These results indicate that the growth supporting activity demonstrated by the IL-1 preparations for tranformed cell lines was not due to contaminating B-cell growth factors (BCGF).

DISCUSSION

A dichotomy has been demonstrated between the ability of affinity-purified IL-1 to influence the DNA response in EBV-transformed B cells and in primary B cells which have been excited through their receptors for antigen. The study was initially undertaken in order to explore the potential role of the cytokine IL-1 in autostimulatory mechanisms of B-cell growth. IL-1 provides a promising candidate as not only has it been shown to be produced by EBV-transformed B cells but has also been proposed to be growth stimulatory for appropriately activated B cells. Nevertheless, discrepancies in the claims for IL-1 involvement in B-cell activation warranted a critical re-examination to the question of its influence in primary B-cell stimulations.

By our rigourous selection of preparations containing only B lymphocytes we feel that we have now dismissed the possibility that IL-1 alone is capable of carrying anti-μ excited cells to DNA synthesis. The discrepancy between our findings and those of Booth & Watson (1984) particularly, probably reflects the purity of the B-cell populations tested.

In contrast to the inability of IL-1 to invoke DNA synthesis in anti-immunoglobulin primed cells, this molecule does appear to play a role in B-cell activation. Furthermore, its presence may be obligatory for cellular replication. Its capacity to provide growth support to EBV-transformed lymphocytes effectively starved of their own autostimulatory factors is consistent with this notion and suggests that IL-1 comprises at least part of the autocrine profile of immortalized B cells. Our observations on primary B-cell cultures suggest that in addition to autoregulatory IL-1 production, EBV-transformed cells must be constitutively expressing at least one other of the growth signals which are normally required for full cell-cycle progression. This could reflect either the production of a second cytokine or, alternatively, the constitutive activation of part of the internal cellular machinery which delivers the growth message to the nucleus. While we have recently shown that cell contact is a crucial factor in generating an optimal growth response in B cells (Gordon, Guy & Walker, 1985), the experiments described in the present paper have been performed under conditions where cell contact has not been limiting and therefore cannot account for the inability of IL-1 to provide a full growth signal for excited B cells.

We are currently exploring in detail the molecules released by EBV transformed cells which are autostimulatory. Preliminary findings indicate a number of species some of which appear to possess classical IL-1 activity and others which lack it. Whether the anti-immunoglobulin co-stimulating activity previously characterized in supernatants from B lymphoblastoid

lines (Gordon *et al.*, 1984b) related to the production of non-IL-1 factors or reflects the synergistic action of B-cell derived IL-1 activities with factors which may have been produced by a few contaminating cells in earlier preparations is also under investigation. The distinct possibility does exist however that the perpetual growth of EBV-transformed cells is a consequence of synergy between an autostimulatory IL-1 and an autostimulatory BCGF. Whatever the outcome of these studies it is clear that the controls on B-lymphocyte growth, and probably cell growth in general, are multiple and complex, presumably reflecting the safeguards necessary to militate against unrestricted proliferations. A precise understanding of the mechanisms by which EBV undermines these controls should prove of considerable interest to those studying the cellular and molecular lesions associated with malignant processes.

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Note added in proof

When assayed for IL-1 activity, crude supernatants from B-cell lines were invariably negative. However, when passed down a G-100 (Pharmacia) column in the presence of a high concentration of polyethylene glycol $(PEG)_{6000}$, fractions containing IL-1 activity were eluted with molecular weights corresponding to 18,000, 35,000 and multiple peaks > 65,000. All these fractions were autostimulatory and, in addition, autostimulatory activity was present with a molecular weight of 45,000–55,000. Some preliminary evidence suggests that this species may possess BCGFII activity.