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The Interleukin-10 Homolog Encoded by Epstein–Barr Virus Enhances the Reactivation of Virus-Specific Cytotoxic T Cell and HLA-Unrestricted Killer Cell Responses

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We determined what influence the Epstein–Barr virus (EBV)-encoded homologue of IL-10 (viral or vIL-10) had on immune responses important for the control of EBV infection. We produced recombinant vIL-10 in a B cell line. A 17-kDa recombinant protein was secreted and had the same molecular weight as vIL-10 secreted by EBV-infected B cells. Functional activity of recombinant vIL-10 was shown by the inhibition of interferon-γ production by activated leukocytes. Cytotoxic T cells and HLA-unrestricted activated killer cells are both important arms of the immune response to EBV. vIL-10, either expressed by B cell stimulators or added exogenously, enhanced the *in vitro* reactivation of allo- and EBV-specific cytotoxic T cells. vIL-10 also enhanced the activation of HLA-unrestricted killer cells by EBV-transformed B cells. In contrast, the interleukin-2-mediated activation of these killers was unaffected. Since vIL-10 is expressed during the lytic cycle of EBV, we conclude that the expression of vIL-10 may enhance immune responses to EBV-infected cells during periods of virus replication *in vivo*. In this way, the virus may limit its own replication and maintain the apathogenic virus carrier state that is characteristic of EBV. © 1992 Academic Press, Inc.

INTRODUCTION

The Epstein–Barr virus (EBV) belongs to a group of herpesviruses which infects humans. Primary infection with EBV usually occurs at an early age and the virus persists for life (Henle and Henle, 1979). The exact mechanism or site of persistence is not clear, although, like most herpesviruses, it involves a combination of both latency and virus replication (Sixbey *et al.*, 1984; Yao *et al.*, 1985, 1989).

There is evidence to suggest that the immune response of the host is important for maintaining the virus–host balance. First, cytotoxic T lymphocytes (CTL) which are specific for EBV-transformed B cells (lymphoblastoid cell lines; LCL) can be reactivated from the peripheral blood of seropositive individuals (Moss *et al.*, 1978; Wallace *et al.*, 1982a,b). Second, HLA-unrestricted activated killer (AK) cells have been implicated in the control of EBV *in vivo* (Rooney *et al.*, 1986). These effectors, which are cytotoxic for EBV-transformed B cells, can also be activated *in vitro* from the peripheral blood (Rooney *et al.*, 1984; Wallace *et al.*, 1982a). Third, individuals with certain types of immunosuppression (e.g., allogeneic organ transplant recipients) can succumb to EBV-associated B lymphoproliferative disease which often regresses once the immunosuppression is relieved (Cleary *et al.*, 1986). Thus, it seems likely that a combination of CTL and AK cells may prevent the spread of EBV from the epithelium and outgrowth of EBV-transformed B cells *in vivo*.

Recently, cDNAs which encode both mouse and human interleukin 10 (IL-10) were cloned (Moore *et al.*, 1990; Vieira *et al.*, 1991). Analysis of these cDNAs revealed that their coding portions had strong homology not only with each other but also with that of an EBV open reading frame termed BCRF1 (using the nomenclature of Baer *et al.*, 1984). Subsequent expression of the BCRF1-encoded protein showed that the protein is similar to human IL-10 in that it is secreted from cells into the medium in a nonglycosylated form and that it has an apparent molecular weight of 17 kDa on SDS–PAGE gels (Hsu *et al.*, 1990). Supernatants containing BCRF1 protein have also been shown to have IL-10 activity in that they block the synthesis of other cytokines, notably interleukin-2 (IL-2) and interferon-γ, from stimulated human peripheral blood mononuclear cells (PBMC: Hsu *et al.*, 1990; Vieira *et al.*, 1991). As a consequence, the BCRF1 protein has been termed viral or vIL-10 (de Waal-Malefyt *et al.*, 1991b) and, in order to avoid confusion, this nomenclature will be used hereafter. Both human and mouse IL-10 have pleiotropic effects, the majority of which are shared by vIL-10. In particular, vIL-10, like human IL-10 downregulates macrophage function by decreasing cytokine synthesis and expression of class II major histocompatibility antigens (de Waal-Malefyt *et al.*, 1991a,b; Fiorentino *et al.*, 1991a,b).

Given that IL-10 has profound effects on cells involved in the immune response, the aim of this work

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was to determine what effect vIL-10 had on types of immunity which are relevant to EBV infection and hence to assess its role in the persistence and pathogenesis of EBV. We therefore expressed vIL-10, using recombinant DNA techniques, in a B cell line and then incorporated either these cells directly or supernatants derived from these cells in reactivation assays *in vitro.* Using these methods, we were able to determine that vIL-10 enhanced the generation of allo-specific CTL, EBV-specific CTL, and HLA-unrestricted AK cells.

MATERIALS AND METHODS

Expression vector construction

The mammalian expression vector (pSNOC) is diagrammatically represented in Fig. 1. BCRF1 was excised as a 935-bp *Stu*I fragment (EBV coordinates 9659–10,594) from cloned EBV DNA (Arrand *et al.,* 1981) and inserted in the appropriate orientation into the *Xba*I site of pSNOC using standard molecular biological techniques (Sambrook *et al.,* 1989). This plasmid construct was termed pSNOC/BCRF1.

BCRF1 was also cloned in the appropriate orientation and frame into the prokaryotic expression vector pGEX-2T (Pharmacia). This vector is designed to drive expression of the inserted sequence fused to glutathione *S*-transferase.

Cells

All cells were maintained, unless specified, at exponential growth in RPMI 1640 (Cellgro, Mediatech) containing 10% fetal calf serum (Cellect Gold; Flow Laboratories, Inc). Certain experiments were performed, where indicated, in AIM-V lymphocyte serum-free medium (GIBCO/BRL).

The B cell line BL41/CL16 was derived from the EBV-negative Burkitt's lymphoma line IARC-BL41 by infection with the defective (EBNA2-deleted) EBV strain HH514.16 (Rooney *et al.,* 1988). The EBV in this line is maintained in a tightly latent form and so expresses EBNA1 but does not produce products associated with virus replication, even after chemical induction with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). The B95-8 cell line is a marmoset LCL which is transformed with the prototypic strain of EBV (Miller *et al.,* 1972). These cells harbor EBV in a latent state but can be induced into productive EBV replication using 20 ng/ml TPA (Bauer, 1983).

The AK-sensitive T cell line HSB-2 was obtained from the American Type Culture Collection (Rockville, MD). The B-LCL, JS/B95, and SV/B95 were derived by infection of PBMC from normal donors with EBV (strain B95-8). The KLOEK cell line (Leger *et al.,* 1987) is an LCL and was obtained from Dr. J. E. Reittie, Royal Free Hospital, London, U.K. The line KW/LCL is an EBV-transformed B cell line which grew spontaneously from the PBMC of a bone marrow transplant recipient (C.M.R., unpublished).

HLA typing

The HLA type of BL41 is A11,32; B35,49 (Rooney *et al.,* 1985) and the HLA type of KLOEK is A2,29; B17,45; DR7 (J. E. Reittie, personal communication). The HLA types of the donor LCL SV/B95 (A2,23; B7,44; DR7,w8), JS/B95 (A1,-; B8,14; DR1,3), and KW/LCL (A2,30; B7,w42; DR11,8) were determined by Dr. E. V. Turner, St. Jude Children's Research Hospital, Memphis.

Transfection of BL41/CL16 cells

BL41/CL16 cells were transfected with either pSNOC alone or pSNOC/BCRF1 by using a Cell-Porator (GIBCO/BRL) as follows: cells were subcultured, the day before transfection, to a density of 4×10^5/ml. The following day, 8×10^6 cells were resuspended in 250 μl of medium and placed in an electroporation cuvette. The appropriate plasmid vector (20 μg) was then added and the cells were exposed to an electrical current using the cell-porator apparatus (250 V at 800 μF). The electroporated cells were then immediately placed in 5 ml medium and cultured for 3 days before being subcultured at a density of 1×10^4 in 1 ml in a 2-ml well of a 24-well plate (Costar) in the presence of 700 μg/ml G418 (Geneticin; GIBCO/BRL). Cells were fed twice per week by the addition of 1 ml of medium containing G418. When growth of cells was detected in the wells (1–2 weeks) cells from one well were taken and subcultured as above. Finally, growth was detected in 6 wells containing cells which had been transfected with pSNOC/BCRF1 and cells expanded from these were termed BC/BL/1 through 6. Likewise, cells which were derived from transfection with pSNOC were termed SN/BL/1. Both sets of cells were routinely maintained in medium containing 700 μg/ml G418 at a density of between 4×10^4 and 4×10^5/ml.

Production of serum-free cell supernatants

Cells were first washed in phosphate-buffered saline, resuspended at 2×10^6/ml in AIM-V serum-free lymphocyte medium (GIBCO/BRL) and incubated for 18 hr, after which time the viability was always >95% as determined by trypan blue staining. At this point the medium was harvested and stored at −70°. Pools of between 70 and 100 ml were concentrated and low molecular weight metabolites removed using Centriprep-10 concentrators (Amicon). Supernatants were

first concentrated 10-fold, then made up to their original volume with RPMI 1640 before being reconcentrated 10-fold.

Production of rabbit monospecific antiserum to vIL-10

The vector pGEX-2T containing BCRF1 (see above) was used to transform *Escherichia coli* BL21. A glutathione *S*-transferase/vIL-10 fusion protein was then expressed in these bacteria according to the manufacturers instructions. Bacteria were lysed directly in SDS–PAGE sample buffer and the lysate electrophoresed through a 10% SDS–PAGE gel (Laemmli, 1970). A band containing the fusion protein was first visualized by adding 5 *M* potassium acetate to the surface of the gel and then excised with a scalpel. The acrylamide gel slice was macerated in phosphate-buffered saline and used to immunize a rabbit. IgG was purified from sera by affinity chromatography using a protein G-sepharose column (MAbTrap G; Pharmacia). The IgG concentration was then quantitated using the optical density at 280 nm.

Radioimmunoprecipitation of vIL-10 from cells

Cells were grown to a density of 5×10^5/ml. Before radiolabeling, cells (1×10^7) were washed twice in methionine-free RPMI 1640 (ICN/Flow) and incubated for 30 mins in 5 ml of this medium. Cells were then pelleted and resuspended in 1 ml of methionine-free RPMI containing 1 mCi of [^{35}S]methionine (cell-labeling grade: NEN). After incubation for 3 hr, the supernatant was collected by centrifugation and diluted 1:1 with RIPA buffer (50 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 0.5% deoxycholate, 1% NP-40, 0.1% SDS, 5% aprotinin, 1 m*M* phenylmethylsulfonyl fluoride). Supernatants were cleared by adding affinity-purified, preimmune IgG (15 μg) to 500 μl of supernatant and incubating for 30 min at room temperature. A volume (50 μl) of 10% fixed *Staphylococcus aureus* Cowan I (Pansorbin; Calbiochem) was then added and the tube incubated for a further 30 min before being centrifuged. The supernatant was collected and incubated again, as above, with Pansorbin. After centrifugation, the supernatant was collected and used for immunoprecipitation. Either preimmune or anti-vIL-10 affinity-purified antibody (15 μg IgG) was added to cleared supernatant (500 μl) and incubated for 2 hr at room temperature. A volume (35 μl) of a 50% solution of protein A sepharose (Pharmacia) contained in 50 m*M* Tris (pH 8.0, 150 m*M* NaCl, 10% bovine serum albumin (fraction V; Boehringer) was then added and the tube incubated for an additional 30 min. Precipitates were collected by centrifugation and washed five times with 1 ml of RIPA buffer. Immunoprecipitated complexes were then solubilized in 35 μl of SDS–PAGE sample buffer and electrophoresed through a 15% SDS–PAGE gel which was subsequently treated with En^{3}hance (DuPont/NEN), dried, and exposed to autoradiographic film. Molecular weight determinations were made using ^{14}C-methylated molecular weight markers (Amersham).

Assay for IL-10 activity

The biological cytokine synthesis inhibitory factor activity of supernatant derived from BC/BL cells were tested in a human IL-10 assay as described previously (Vieira *et al.*, 1991). Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood on Ficoll–Hypaque as described (Boyum, 1968) and were stimulated with phytohemagglutinin (0.5 μg/ml) in AIM-V medium in the presence of varying amounts of supernatant. After 3 days, the medium was harvested and assayed for interferon-γ using a commercial ELISA (GIBCO/BRL). One unit of IL-10 activity is defined as 50% of maximum inhibition of interferon-γ synthesis.

Reactivation of CTL

PBMC (see above) were cocultured at a concentration of 2×10^6/2 ml/well in 24-well plates with 5×10^4 γ-irradiated (4000 rad) stimulator cells (responder:stimulator ratio of 40:1) as described previously (Wallace *et al.*, 1982a). The cells were fed by renewing half the volume of medium after 5 days and after 14 days, the cells were harvested and used as effectors in a chromium release assay.

Induction of activated killer cells with IL-2

PBMC were cultured overnight in AIM-V serum-free medium in the presence of 1000 U/ml recombinant IL-2 (Boehringer) at a density of 2.5×10^6/ml. Cells were then harvested and incorporated as effectors in a chromium release assay.

Induction of activated killer cells with autologous LCL

PBMC were cocultured at a density of 2×10^6 per 2-ml well along with 5×10^5 γ-irradiated autologous LCL (4:1 responder:stimulator ratio) as described by Wallace *et al.* (1982a). After culture for 7 days, the cells were harvested and used as effectors in a cytotoxicity assay.

Chromium release assays

Chromium release assays were performed as described previously (Wallace *et al.*, 1982b). Briefly, target cells (1×10^6) were labeled with ^{51}Cr (100 μCi in

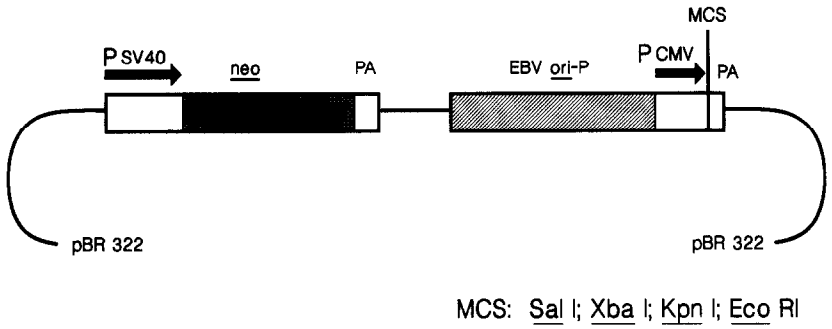

FIG. 1. Diagrammatic representation of the mammalian expression vector pSNOC. The construction of this plasmid was performed by C. Rooney and G. Allan (unpublished data). Bacterial plasmid sequences are represented by lines and regions of the vector relevant to expression in mammalian cells are represented by open boxes. This vector is based upon the bacterial cloning vector pBR322. The neomycin gene (*neo*), expression of which is driven by the SV40 promoter (PSV40), enables selection of cells containing the plasmid by the use of G418. The presence of the EBV *ori*-P element enables the vector to be maintained as an episome in cells which express EBNA1 (Yates *et al.*, 1984). Expression of the gene of interest is driven by the cytomegalovirus immediate–early promoter-enhancer element which is contained upstream of unique multiple cloning sites (MCS) which in turn are upstream of the SV40 polyadenylation signal sequences (PA).

200 μl for 1 h) before being washed 5 times. Labeled target cells (5×10^3) were mixed with effector cells at appropriate ratios in a final volume of 200 μl in the wells of a round-bottomed 96-welled plate. Where appropriate, the targets were incubated for 30 min with 6 μg of monoclonal antibody before inclusion in the assay. The reagents used were the anti-HLA class I monoclonal antibody W6/32 (Dakopatts) or an isotype-matched dengue virus-specific monoclonal antibody HB114 (a kind gift of Dr. J. E. Allan). After incubation for 5 hr, supernatants (100 μl) were harvested and counted using a gamma counter (Cobra autogamma). Specific cytotoxicity was worked out by using the formula: percentage specific cytotoxicity = experimental release − spontaneous release/maximum release − spontaneous release × 100.

Lytic units

Differences between the activities of effector populations in a given experiment were quantitated by using lytic units. Lytic units were calculated as described by Kay (1980). The percentage specific cytotoxicity was plotted versus the log of the various effector:target ratios. Cytotoxicity curves were then generated from which the number of effector lymphocytes required to produce 30% specific cytotoxicity were calculated. This number of lymphocytes represents one lytic unit (LU) in that experiment. Results were expressed as lytic units per 10^6 cells.

RESULTS

Stable expression of vIL-10 in B cells

The EBV BCRF1 open-reading frame which encodes vIL-10 was inserted into the vector pSNOC (Fig. 1). This plasmid was transfected into BL41/CL16 cells. BL41/CL16 cells were chosen for several reasons. First, they do not express detectable levels of either human IL-10 or vIL-10 mRNA by Northern blotting (J.P.S., manuscript in preparation). Second, by virtue of being EBV-positive, they express EBNA1 and so are able to maintain pSNOC as an episome. Finally, B cells are a natural host for EBV and we wished to retain a relatively authentic cellular background.

After transfection with pSNOC/BCRF1, six G418-resistant lines were obtained. In an initial screen, all six lines, termed BC/BL/1–6, were found to express equal amounts of vIL-10 mRNA, while control cells transfected with pSNOC alone (SN/BL/1) expressed none.

To determine whether recombinant vIL-10 of the appropriate molecular weight was secreted into the medium, supernatants derived from transfected cells and EBV-transformed cells were radioimmunoprecipitated using rabbit monospecific IgG to vIL-10. Figure 2 shows the results of these experiments. Figure 2A shows that a protein of apparent molecular weight 17 kDa was precipitated from BC/BL/5 cell supernatants using anti-vIL-10 (lane 1). This protein was not precipitated from BC/BL/5 supernatant using preimmune IgG or from the control SN/BL/1 supernatant using either anti-vIL-10 or preimmune IgG (lanes 2–4). In additional experiments, the lines BC/BL/1 and BC/BL/6 were also shown to secrete recombinant vIL-10 of the same molecular weight. Thus, expression of vIL-10 mRNA by the transfected lines was paralleled by secretion of recombinant protein into the medium. The molecular weight of our product was the same as had been previously reported for recombinant vIL-10 expressed in COS cells (Hsu *et al.*, 1990). The experiment shown in panel B compares proteins immunoprecipitated from supernatants of the EBV-transformed line B95-8 with

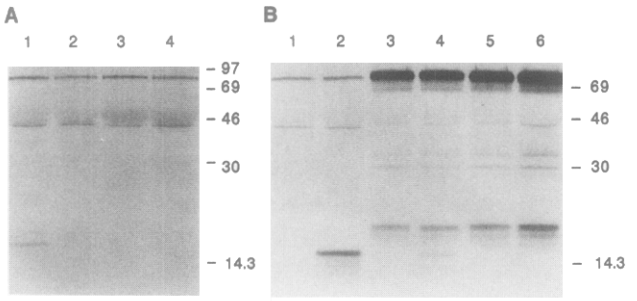

FIG. 2. Radioimmunoprecipitation of vIL-10 from transfected BL41/CL16 cells and EBV-transformed (B95-8) cells. Radiolabeled proteins in the supernatants of cells were immunoprecipitated with either monospecific rabbit IgG to vIL-10 or preimmune IgG from the same rabbit. Precipitated proteins were then analyzed by SDS–PAGE and autoradiography. The positions of molecular weight standards ($\times 10^{-3}$ Da) are shown at the right. (A) Expression of recombinant vIL-10 by transfected BL41/CL16 cells. Lanes 1 and 3 show precipitations with anti-vIL-10 IgG, whereas lanes 2 and 4 show precipitations with preimmune IgG. Precipitation was performed with either BC/BL/5 supernatant (lanes 1 and 2) or SN/BL/1 supernatant (lanes 3 and 4). (B) Comparison of the expression of recombinant and authentic vIL-10. Lanes 1, 3, and 5 show precipitations with preimmune IgG, whereas lanes 2, 4, and 6 show precipitations with anti-vIL-10 IgG. Precipitation was performed with supernatants derived from either BC/BL/5 cells (lanes 1 and 2), B95-8 cells induced into productive replication with TPA (lanes 3 and 4), or untreated B95-8 cells (lanes 5 and 6).

those from transfected cells. As in Fig. 2A, a 17-kDa protein corresponding to vIL-10 was precipitated from BC/BL/5 cell supernatants using anti-vIL-10 IgG (lane 2) but not preimmune IgG (lane 1). When supernatants of B95-8 cells were used, a 17-kDa protein was precipitated with anti-vIL-10 only when the EBV productive cycle was induced in these cells using TPA (lane 4). No protein of this molecular weight was precipitated from the supernatants of induced B95-8 cells using preimmune IgG (lane 3) or from the supernatants of untreated B95-8 cells using either anti-vIL-10 (lane 6) or preimmune IgG (lane 5). Therefore, recombinant vIL-10 has the same molecular weight as the authentic protein produced by B95-8 cells. This data also adds to mRNA expression studies (Vieira *et al.*, 1991) by demonstrating for the first time that vIL-10 protein is secreted by infected B cells during the lytic but not the latent cycle of EBV.

To determine whether the supernatants derived from BC/BL cells had functional IL-10 activity, we performed a human cytokine synthesis inhibitory factor assay as described by Vieira *et al.* (1991). The amount of human interferon-γ produced by phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMC) was measured in the presence of supernatants derived from either BC/BL or SN/BL cells. As can be seen in Fig. 3, supernatant from SN/BL/1 cells did not decrease the synthesis of interferon-γ. However, BC/BL supernatant caused decreased interferon-γ synthesis. This effect was abrogated by the addition of rabbit anti-vIL-10 IgG but was unaffected by the addition of preimmune IgG. Thus, the inhibition observed was a direct effect of vIL-10 and not due to the action of other cytokines present in the supernatant. In titration experiments, the level of IL-10 activity in 10-fold concentrated BC/BL supernatants was consistently found to be around 100 U/ml, where one unit is the amount required to cause 50% of the maximum decrease in interferon-γ synthesis.

The above sets of results show that we had generated B cells which secreted functional recombinant vIL-10 as a source of the factor for use in cytotoxic cell reactivation experiments.

Effect of vIL-10 on the generation of CTL responses

To ascertain the effect of vIL-10 on the generation of CTL responses we first used the *in vitro* reactivation of allo-specific CTL as a model. This enabled us to use the transfected BL41/CL16 cells as stimulators and therefore compare the effect of vIL-10 expression by the antigen-presenting cell itself with the effects of exogenously added factor.

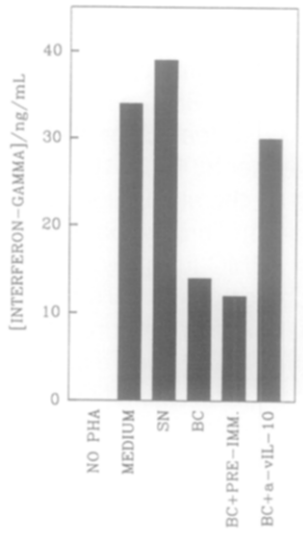

FIG. 3. Effect of vIL-10 on the production of interferon-γ by activated PBMC. The amount of interferon-γ produced by phytohemagglutin-stimulated PBMC are shown by bars. Each bar represents the mean of three determinations. Interferon-γ produced by unactivated PBMC was added as a control (NO PHA). Activation was performed either medium alone (MEDIUM), medium containing 10% 10-fold concentrated SN/BL/1 supernatant (SN), or medium containing an equivalent amount of BC/BL/5 supernatant (BC). In addition, IgG (30 μg/ml), either preimmune (BC + PRE-IMM) or anti-vIL-10 (BC + a-vIL-10), was included in activations performed with BC/BL/5 supernatant.

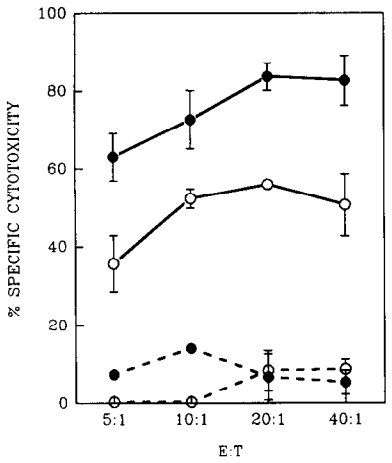

FIG. 4. Effect of vIL-10 expression reactivation of an allo-specific CTL response. BC/BL/5 cells expressing vIL-10 or control SN/BL/1 cells as stimulators were cocultured with allogeneic PBMC (donor J.S.) at a responder:stimulator ratio of 40:1. Effector cells were tested against either labeled BL41/CL16 or HLA-mismatched LCL in a chromium release assay. The results are plotted as specific cytotoxicity observed at four effector:target (E:T) ratios. Results from experiments where BC/BL/5 cells were used as stimulators (●) and those where SN/BL/1 cells were used as stimulators (○) are plotted on the same graph. The results from using BL41/CL16 as targets are represented by solid lines and those from using HLA-mismatched LCL (SV/B95) by broken lines. The standard deviation from the mean of triplicates are shown by bars.

Allo-specific CTL were generated by coculturing either BC/BL/5 (expressing vIL-10) or SN/BL/1 (vector control) cells as the stimulators (antigen-presenting cells) with allogeneic PBMC as the responders. Effector populations were tested for allo-specific cytotoxic activity against either BL41/CL16 cells, which have the same HLA type as the stimulator cells or an HLA-mismatched LCL target. Three separate experiments were performed with PBMC from two donors with identical results. The number of live cells harvested varied between individual experiments from $0.8–1.2 \times 10^6$/ml but was the same in any given experiment irrespective of the stimulator. The results of one representative cytotoxicity assay are shown in Fig. 4. Effectors derived by coculture with BC/BL/5 cells showed a significantly elevated lysis of BL41/CL16 targets as compared with effectors which were generated by coculture with SN/BL/1 cells. The lysis of BL41/CL16 cells by effectors in both cases was blocked (30%) by the addition of the anti-HLA class I monoclonal antibody W6/32 but was completely unaffected by the addition of an isotype-matched, negative control monoclonal antibody, HB114. This, taken with the fact that HLA-mismatched targets were not killed, demonstrates that the killing observed was mediated by classical HLA class I-restricted CTL. The elevated cytotoxic activity of effectors generated using BC/BL/5 cells was quantitated by calculating lytic units (LU) and was found to be approximately seven times greater than those generated using SN/BL/1 cells (400 versus 60 LU/10^6 cells, respectively). Both sets of effectors were also tested against BC/BL/5 and SN/BL/1 cells in order to see whether vIL-10 expression altered the sensitivity of B cells to lysis by CTL. However, the levels of killing, in both cases, were almost identical to BL41/CL16 targets.

Additional reactivation experiments were performed using SN/BL/1 cells as stimulators and 10-fold concentrated supernatant from BC/BL/5 as a source of exogenous vIL-10. Like those derived using BC/BL/5 cell stimulators, effectors derived in this fashion displayed an elevated level of allo-specific cytotoxicity as compared with reactivations performed using control (SN/BL/1) supernatant (not shown). Thus, exogenous vIL-10 was also able to elevate the reactivation of CTL killing.

To determine the effect of vIL-10 on the generation of an EBV-specific CTL response, we cocultured EBV-transformed B cells (LCL) derived from an EBV-seropositive donor (SV) along with autologous PBMC in the presence of either BC/BL/5 or SN/BL/1 cell supernatant. The number of live cells harvested from the cocultures were identical. The results of cytotoxicity assays (Fig. 5) showed that an autologous LCL (Fig. 5A) was lysed to a greater extent than an LCL which shared two HLA class I haplotypes (Fig. 5B) and that HLA mismatched LCL (panel C) was not lysed at all. The amount of cytotoxicity observed was significantly enhanced when the effector populations were reactivated in the presence of vIL-10-containing BC/BL/5 supernatant. HLA class I restriction of the effectors was confirmed by blocking lysis of autologous LCL with W6/32 monoclonal antibody (shown by bars). The degree of elevation of EBV-specific CTL activity by the addition of vIL-10 as compared to the control was quantitated by calculating LU and was found to be threefold for the autologous target (110 versus 30 LU/10^6 cells, respectively) and fourfold for the hemiallogeneic target (18 versus 4.5 LU/10^6 cells, respectively). These results demonstrate that vIL-10, either expressed by the stimulator B cells or added exogenously, enhanced the *in vitro* reactivation of both allo- and EBV-specific, HLA class I-restricted CTL.

Effect of vIL-10 on the generation of activated, HLA-unrestricted killer cells

Previous studies have suggested that activated, HLA-unrestricted killer (AK) cells are important for the control of EBV infection *in vivo* (Rooney *et al.*, 1986). To determine what effect vIL-10 had on the generation of

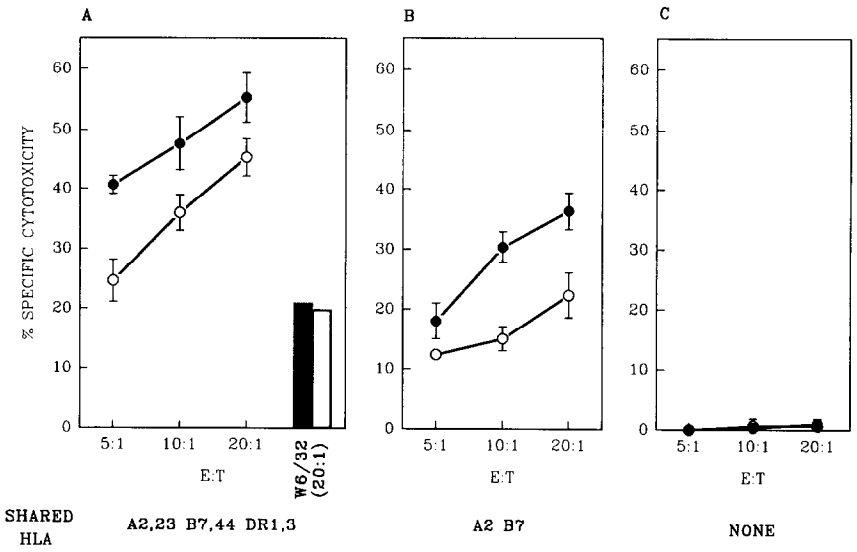

FIG. 5. Effect of vIL-10 on the reactivation of EBV-specific CTL. LCL (SV/B95) were cocultured with autologous PBMC at a responder:stimulator ratio of 40:1 with the addition of 10% concentrated supernatant from either BC/BL/5 or SN/BL/1 cells. Effector cells obtained were tested against either labeled autologous LCL (SV/B95; A), HLA partially matched LCL (KW/LCL; B) or HLA-mismatched LCL (JS/B95; C) in a chromium release assay. The HLA haplotypes which are shared with the effector population are indicated below the panels. The results are plotted as specific cytotoxicity observed at three effector:target (E:T) ratios. The cytotoxicity observed from effectors reactivated in the presence of BC/BL/5 supernatant containing vIL-10 (●) and those reactivated in the presence of control SN/BL/1 supernatant (○) are plotted on the same graph. The standard deviation from the mean of triplicates are shown by bars. The results of blocking HLA class I-mediated killing of autologous LCL using the monoclonal antibody W6/32 at an effector:target (E:T) of 20:1 are represented as bars at the left of (A): values from effectors reactivated in the presence of BC/BL/5 (■) and SN/BL/1 (□) are shown side by side.

such cells *in vitro* we activated HLA-unrestricted cells from the PBMC of donors using either interleukin-2 (IL-2) or an autologous LCL at a high stimulator:responder ratio (Wallace *et al.*, 1982). Effector cells were then assayed for cytotoxicity against EBV-negative AK-sensitive cells (HSB-2) and an HLA-mismatched LCL (KLOEK).

AK cells were reactivated by IL-2 on three separate occasions with identical results. The number of cells harvested was identical to the input number in all cultures. The results of one representative experiment are shown in Fig. 6. HSB-2 cells (Fig. 6A) were insensitive to lysis by unstimulated PBMC. After addition of IL-2, the effector cells lysed HSB-2, but the cytotoxic activity was not significantly different whether IL-2 plus SN/BL/1 supernatant or IL-2 plus BC/BL/5 supernatant were used in the activation. Figure 6B shows that the pattern of lysis of KLOEK LCL, by all effector populations, was similar to that seen with HSB-2.

Next, we assessed the effect of vIL-10 on the generation of AK cells by an autologous LCL. Effectors were reactivated in this fashion on three separate occasions with identical results. The number of live cells harvested from the cocultures was the same in any given experiment whether BC/BL/5 supernatant or SN/BL/1 supernatant was used. The results of one representative experiment are shown in Fig. 7. Stimulation with an LCL-induced effector populations which lysed HSB-2 (Fig. 7A) and KLOEK LCL (Fig. 7B). However, in contrast to IL-2 activation, the level of killing of both targets was significantly enhanced when the stimulation was performed in the presence of vIL-10-containing BC/BL/5 supernatant. The degree of elevation of cytotoxicity by the addition of vIL-10 as compared to the control was quantitated by calculating LU and was found to be four times greater with HSB-2 targets (285 versus 80 LU/10^6 cells, respectively) and five times greater with KLOEK LCL targets (333 versus 60 LU/10^6 cells, respectively). Thus, vIL-10 enhanced the *in vitro* reactivation of AK cells capable of lysing EBV-transformed B cells but only when the induction was mediated by virus-transformed B cells.

DISCUSSION

We have described the effects of recombinant vIL-10 on the *in vitro* reactivation of immune cytotoxic cells which are thought to be important for the control of EBV *in vivo*. Using recombinant vIL-10 expressed in a transfected B cell line, we demonstrated that vIL-10, either expressed by B cell stimulators or added exogenously, enhanced the *in vitro* reactivation of allo- and EBV-specific, HLA-restricted CTL. In a similar fashion, vIL-10 increased the reactivation of HLA-unrestricted

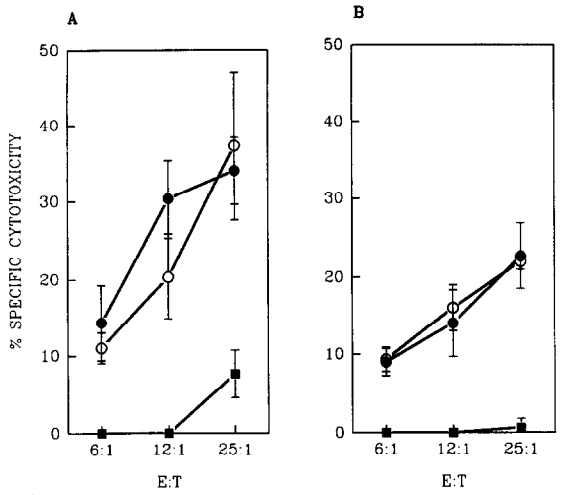

FIG. 6. Effect of vIL-10 on the IL-2-mediated induction of AK cells. PBMC (donor J.S.) were stimulated with IL-2 in combination with 10% 10-fold concentrated supernatant derived from BC/BL/5 or SN/BL/1 cells. Unstimulated PBMC were added as a control. Effector cells were tested against labeled target cells in a chromium release assay. The results are plotted as specific cytotoxicity at different effector:target (E:T) ratios. Results obtained with unstimulated cells (■), cells stimulated with IL-2 plus control SN/BL/1 supernatant (○), and cells stimulated with IL-2 plus vIL-10-containing BC/BL/5 supernatant (●) are shown on the same graph. The target cells used were HSB-2 (A) and KLOEK LCL (B). The standard deviation from the mean of triplicates are shown by bars.

AK cells when these were generated *in vitro* using autologous LCL, although it had no effect on the IL-2-mediated generation of these effectors. In addition, we showed that authentic (nonrecombinant) vIL-10 was secreted from transformed B cells during the lytic but not the latent cycle of EBV.

The effect of vIL-10 on cytotoxicity is somewhat paradoxical. Previous effects of vIL-10, like human IL-10, have been inhibitory to immune responses. In particular, vIL-10 decreases the production of a range of proinflammatory cytokines (e.g., IL-1α, IL-1β, IL-6, IL-8, tumor necrosis factor α, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor) from activated macrophages (de Waal-Malefyt *et al.*, 1991a; Fiorentino *et al.*, 1991a). Both human and viral IL-10 have also been shown to decrease the expression of class II major histocompatibility molecules on macrophages which in turn renders them incapable of antigen-presenting cell-dependent production of IL-2 and interferon-γ by T helper type 1 cells (de Waal-Malefyt *et al.*, 1991b; Fiorentino *et al.*, 1991b; Taga and Tosato, 1992).

vIL-10 may increase the lytic activity of the CTL and AK cell effector populations by a number of mechanisms. One possibility is that vIL-10 upregulates antigen presentation by B lymphocytes, although current evidence suggests that this is not the case (de Waal-Malefyt *et al.*, 1991b). Alternatively, although total cell numbers were not increased, vIL-10 may act on T cells in a similar manner to mouse IL-10 on murine T cells (Chen and Zlotnik, 1991) and selectively expand cytotoxic cell subsets.

The two methods of AK cell generation used here (IL-2 and autologous LCL) resulted in functionally similar effector populations. However, they differed in their sensitivity to upregulation by vIL-10, suggesting that the two methods represent either the stimulation of different AK cell types or the use of two distinct activation pathways. It has been suggested that the generation of AK cells by virus infections *in vivo* depends on cytokines other than IL-2 (Welsh *et al.*, 1991). This, along with the fact that the induction of AK cells with an LCL is an EBV-activated response, suggests that vIL-10 might enhance an AK response to EBV-infected cells *in vivo*.

Infection with EBV is generally apathogenic: the virus has evolved a relationship with the host where a low level of chronic virus replication and latency without disease are found. How does vIL-10 contribute to this pattern of behavior? A high level of EBV replication with associated disease is only detected for a limited

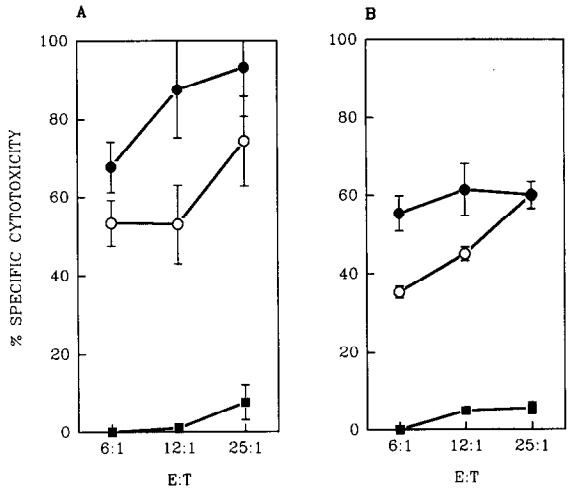

FIG. 7. Effect of vIL-10 on the stimulation of AK cells using autologous LCL. PBMC (donor J.S.) were stimulated with an autologous LCL at a responder:stimulator ratio of 4:1 plus 10% 10-fold concentrated supernatant derived from either BC/BL/5 or SN/BL/1 cells. Unstimulated PBMC were added as a control. Effector cells were tested against labeled target cells in a chromium release assay. The results are plotted as specific cytotoxicity observed at different effector:target (E:T) ratios. Results obtained with unstimulated cells (■) cells stimulated in the presence of control SN/BL/1 supernatant (○), and cells stimulated in the presence of vIL-10-containing BC/BL/5 supernatant (●) are shown on the same graph. The target cells used were HSB-2 (A) and KLOEK LCL (B). The standard deviation from the mean of triplicates are shown by bars.

period after primary infection and appears to be controlled by a vigorous immune response (Strang and Rickinson, 1987). Unlike B cells transformed by EBV *in vitro,* there is evidence to suggest that normally, *in vivo,* latently-infected B cells have a more restricted pattern of EBV gene expression and a less activated cell phenotype which are similar to freshly explanted Burkitt's lymphoma cells (Rowe *et al.,* 1992). Cells with this phenotype, termed "group I," are also relatively insensitive to lysis by cytotoxic cells (Rooney *et al.,* 1984, 1985). We have shown that vIL-10 protein, like mRNA, is expressed during the productive, but not the latent cycle of EBV infection. Since this protein may upregulate CTL and AK cells which are the most important control mechanism for EBV *in vivo,* production of vIL-10 may be a mechanism which the virus has evolved to enhance an immune response to itself, causing the selection of latently infected cells with a group I phenotype. These cells would not express vIL-10 and would also be poorly recognized by cytotoxic cells. In this way, the virus may limit its own replication and may maintain the apathogenic virus carrier state that is characteristic of EBV.

vIL-10 may contribute in other ways to the life cycle of the virus. Recently, it was shown that vIL-10 is also a B cell growth and differentiation factor (Defrance *et al.,* 1992; Rousset *et al.,* 1992), hence it could enhance the infection of B cells by EBV. This pleiotropic protein might therefore play an number of important roles in virus infection and its future study may provide valuable insight into the mechanisms of EBV persistence and tumorigenesis.

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