Epstein-Barr Virus-Transformed B Cells, a Potentially Convenient Source of Autologous Antigen-Presenting Cells for the Propagation of Certain Human Cytotoxic T Lymphocytes

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Received 28 February 1994/Returned for Modification 13 April 1994/Accepted 21 July 1994

Antigen-specific cytotoxic T cells (CTL) are generally elicited in vitro by incubation of effector cells with an appropriate major histocompatibility complex-matched antigen-presenting cell (APC). In the case of CTL derived from inbred rodents, spleen cells from an animal of the same strain serve as a ready source of autologous major histocompatibility complex-identical APC. In outbred human populations, however, a convenient source of human leukocyte antigen-matched APC is ordinarily difficult to obtain, and for that reason human antigen-specific CTL may be difficult to propagate. We describe a method whereby Epstein-Barr virus-transformed human B cells (B-LCL) serve as a convenient source of efficient APC for the propagation of human antigen-specific CTL. B-LCL are produced by using B cells from the donor under study and are thus human leukocyte antigen identical to the donor. Using this method, we have propagated human CD4+ Toxoplasma gondii-specific CTL for up to 9 months in vitro, during which time the cells retained their functional capability.

Following intracellular processing, antigen is presented to cytotoxic T lymphocytes (CTL) in the context of the appropriate major histocompatibility complex molecule in vivo by professional antigen-presenting cells (APC), consisting predominantly of monocytes and macrophages (10, 22). However, many cell types may present antigen under appropriate conditions. Spleen cells are often used as a convenient, plentiful source of major histocompatibility complex-identical APC for in vitro stimulation of CTL in rodents. Work with human CTL, on the other hand, may be hampered by the lack of a ready supply of autologous HLA-matched APC for restimulating and propagating these cells. One must often rely on the same subject to make repeated donations of blood as the source of autologous APC. However, if the donor becomes gravely ill, moves, dies, or refuses to participate further in studies, the CTL may be lost for lack of appropriate means to restimulate them.

Epstein-Barr virus (EBV)-transformed human B lymphocytes (B-LCL) have been used extensively as autologous target cells for the study of cytolysis mediated by human antigen-specific CTL (4, 6, 7, 13, 16, 20, 24, 29, 33, 34). These transformed B cells are capable of presenting antigen in the context of both HLA class I and class II molecules (4, 6, 7, 13, 16, 20, 24, 29, 33, 34). Their use as APC to stimulate proliferative responses has also been extensively described (2, 3, 8, 9, 17). A relatively small number of studies have demonstrated that these cells may also be useful as APC for the stimulation of human CTL (6, 14, 30), but the technique is not widely appreciated. We describe our experience with the use of these cells as APC for the purpose of stimulating human CD4+ Toxoplasma gondii-specific CTL and briefly review the literature on the use of B-LCL as APC for stimulation of antigen-specific T cells. Although our present experience deals with CD4+ CTL, published experience suggests that this method is applicable to certain human CD8+ CTL as well.

MATERIALS AND METHODS

Human subjects. Subjects were apparently healthy humans with serologic evidence of prior infection with T. gondii. The study was approved by the Institutional Review Board of the University of Colorado Health Sciences Center, and all subjects gave written informed consent.

Cell lines and antigenic preparations. B95-8, an EBV-producer cell line, and human foreskin fibroblasts were obtained from the American Type Culture Collection. Toxoplasma gondii RH was originally the gift of Elmer Pfefferkorn. EBV transformation of human B cells was performed by a well-described method (36). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density centrifugation. A total of 2 x 10⁶ cells were suspended at 2 x 10⁶ cells per ml in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) buffer, 50 U of penicillin per ml, 50 μg of streptomycin per ml, 20% heat-inactivated fetal calf serum (Sigma, St. Louis, Mo.), 20% supernatant from the B95-8 cell line, and 50 nM cyclosporin A (Sandoz, Charloetteville, N.C.). The cells were fed weekly with exchanges of growth medium, prepared as above with 10% heat-inactivated fetal calf serum (with no further cyclosporin A or B95-8 supernatant) until transformation was apparent 3 to 10 weeks later. At that time, cells were fed twice weekly with growth medium containing 10% fetal calf serum and maintained at approximately 3 x 10⁶ to 7 x 10⁶ cells per ml for optimal growth. Transformed cells were termed B-LCL. Toxoplasma antigen and control human fibroblast antigen were prepared from freeze-thawed cells as described previously (6).

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APC. To produce antigen-pulsed APC, B-LCL or PBMC were incubated with 1 to 10 μg of soluble protein antigen in 1 ml of growth medium at a density of 1 × 10^6 to 2 × 10^6 cells per ml at 37°C, in a humidified 5% CO2 atmosphere. Following overnight incubation (unless otherwise specified) to allow for antigenic processing, APC were then exposed to a cobalt gamma irradiation source to inhibit further growth. To produce infected APC, B-LCL were incubated overnight with Toxoplasma tachyzoites, incubated with pyrimethamine, and irradiated as described previously (6). Infected APC which had not been treated with pyrimethamine but were simply irradiated with as much as 18,000 rads of gamma irradiation were found to be unsuitable, owing to outgrowth of tachyzoites in culture (our unpublished observations).

Cell counts and irradiation. Cells were irradiated in 1-ml volumes by exposure to a cobalt source and then suspended in phosphate-buffered saline and filtered Trypan blue dye for enumeration and determination of viability. All datum points were determined in triplicate, and the mean result was reported.

CTL. Human CD4+ T. gondii-specific CTL clones used for these experiments have been reported on previously (6), and were designated T007.1 to T007.17 in that study. They were produced as follows. PBMC from human donors were incubated for 10 days with antigen derived from T. gondii at 10 μg/ml. Stimulated cells were then seeded at limiting dilution into 96-well flat-bottomed tissue culture plates in the presence of irradiated (3,000 rads) autologous PBMC at 5 × 10^5 cells per ml as a source of feeder cells and irradiated (3,000 rads) autologous antigen-pulsed PBMC at 3 × 10^4 cells per ml as APC in growth medium containing 50 U of recombinant human interleukin-2 (Hoffman-La Roche, Nutley, N.J.) per ml. Wells were observed for growth, which was usually evident by 2 weeks following seeding. At about 4 to 5 weeks following seeding, cells from wells exhibiting greater than 50% confluent growth were transferred to 24-well plates. Cells in 96-well plates were fed with partial medium exchanges weekly during this observation period and were not restimulated until transfer to 24-well plates. Thereafter, CTL were propagated by restimulation with autologous, antigen-pulsed B-LCL as described above and with allogeneic feeder cells (10^6/ml) approximately every 2 weeks.

Allogeneic feeder cells kept frozen at −70°C for up to 6 months were used successfully in restimulations (our unpublished observations), further adding to the simplicity and convenience of this system. B-LCL were not used initially as APC, because they induce EBV-specific CTL (21, 27, 28, 35 and our unpublished results). Cell surface phenotypes of the CTL clones were determined with a Coulter EPICS Profile II as previously described (6).

Cytotoxicity assay. The methods used were described previously (6, 7). Briefly, effector cells and 51Cr-labeled target cells were incubated together in a total volume of 200 μl for 4 h, unless otherwise indicated. Cytotoxicity was determined by 51Cr release. All datum points were determined in triplicate, and the mean result was reported. Results of experiments in which the spontaneous release of 51Cr by target cells exceeded 30% of the maximum release were discarded.

RESULTS

Radiation susceptibility of B-LCL. To ensure that B-LCL in culture did not continue to grow, a radiation dose-response curve for these cells was established. B-LCL were irradiated with 1,000 to 6,000 rads, resuspended at 5 × 10^5 cells per ml, and returned to culture. We determined that 3,000 to 6,000 rads reliably killed B-LCL (results not shown); therefore, for all work described herein, B-LCL were killed by irradiation with 6,000 rads. Previous work (33, 34) has demonstrated the susceptibility of PBMC to irradiation. We have also demonstrated that T-cell clones produced by stimulation with irradiated B-LCL were uninfected by residual B cells (6).

Antigen-pulsed B-LCL as APC. B-LCL were incubated with T. gondii antigen or control human fibroblast antigen at 10 μg/ml for various times and then used as target cells for the clone T007.17. By time course analysis, we determined that antigen processing by B-LCL for this protein mix was near maximal between 16 and 40 h following pulsing (Fig. 1). For convenience, we therefore chose overnight incubation of APC with antigen prior to irradiation. However, APC incubated with Toxoplasma antigen for as little as 1 h efficiently restimulated these CD4+ CTL (not shown). A similar time course for antigen processing by PBMC was not performed owing to the heterogeneity of these cells and the difficulties in their use as CTL target cells (our unpublished data).

CTL propagated with B-LCL as APC. The method described herein allowed for propagation of CD4+ Toxoplasma-specific CTL for up to 9 months in culture without loss of antigenic specificity, although cytolytic capacity diminished over time (Table 1). CTL survived in culture for an average of approximately 6 months (range, 2.5 to 13 months). Although an internal positive CTL control was not available to standardize cytolyis, the loss of cytolytic activity was not simply variation in our assay, because no cytolyis was detectable by 11 months following original seeding in the CTL reported here (Table 1).

Cells initially grew vigorously, doubling approximately every 1 to 1.5 days following transfer to 24-well plates. By 6 months in culture, most surviving CTL grew more slowly, doubling approximately every 2.5 to 3 days. Growth progressively slowed until all CTL eventually died. Fluorescence-activated cell sorter analysis demonstrated that the CTL were ≥95% CD3+, CD4+, and CD8−, including at the latest time points when cytolyis was no longer evident and cells were dying (6; our unpublished observations).
TABLE 1. Human CD4+ Toxoplasma-specific CTL retain their functional capacity for up to 9 months in culture, although their activity diminished over time

<table>
<thead>
<tr>
<th>Clone</th>
<th>% Specific cytolysis after culture³:</th>
<th>3 mo</th>
<th>4 mo</th>
<th>9 mo</th>
<th>11 mo</th>
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<td>79</td>
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<td></td>
<td>46</td>
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</table>

¹ These clones have been described in detail elsewhere (6). Numbers 1 to 10 refer to clones T007.1 to T007.10, respectively.
² Assays were done at an effectorto-target-cell ratio of 3:1. Maximum ³¹Cr release for the target cells used in these experiments was 5,560 cpm at month 3, 6,179 cpm at month 4, 6,632 cpm at month 8, and 7,468 cpm at month 11.
³ ND, not done, because the cell culture had died.

Initial stimulations of PBMC and restimulations of CTL transferred into 24-well plates were done with APC/PBMC or APC/CTL ratios of approximately 1:10. An APC/CTL ratio of 1:4 was also tested. However, as this density of B-LCL inhibited established CTL clone growth and occasionally caused death of the culture (data not shown), it was not used further. APC/CTL ratios of as low as 1:40 also successfully restimulated established CTL clones, but no systematic study was performed. The decision to use an APC/CTL ratio of 1:10 was thus somewhat arbitrary.

These CTL clones, propagated with allogeneic feeder cells and B-LCL, were neither allospecific nor EBV specific, as they did not lyse allogeneic B-LCL (Fig. 2 and data not shown) or autologous B-LCL which did not express Toxoplasma antigens (Fig. 1 to 3 and data not shown).

To assess whether B-LCL would serve as APC at the time of initial stimulation of effector cells, freshly isolated PBMC were incubated with autologous Toxoplasma antigen-pulsed B-LCL at an APC/PBMC ratio of 1:10. At 10 days later, cells were subcloned at limiting dilution with irradiated, autologous PBMC as feeder cells and autologous, irradiated, antigen-pulsed B-LCL as APC. In the single subject tested in this manner, derived CTL were exclusively CD4+ and EBV specific. No Toxoplasma-specific CTL were detected (data not shown). We did not study the use of allogeneic feeder cells at the initial stimulation, because this method probably would have resulted in the production of allospecific CTL.

Human CD8+ Toxoplasma-specific CTL were not readily generated by this system (6). Although neither tachyzoite-infected B-LCL nor PBMC were able to elicit CD8+ CTL, they served as target cells for CD4+ CTL in cytotoxicity assays (Fig. 3) and elicited CD4+ Toxoplasma-specific CTL (6). We are accumulating evidence that Toxoplasma-infected B-LCL and PBMC express Toxoplasma antigens poorly to human CD8+ cells (unpublished data). As B-LCL served as APC for CD8+ CTL of other specificities (4, 13–16, 20, 24, 30, 33, 34, 37), the relative inefficiency of infected B-LCL to produce Toxoplasma-specific CD8+ CTL appears to reside in properties intrinsic to infection with the parasite rather than to the B-LCL.

**DISCUSSION**

The system we now describe allowed for convenient propagation of human CD4+ Toxoplasma-specific CTL. PBMC were initially stimulated with autologous, antigen-pulsed, irradiated PBMC as APC in the presence of autologous, irradiated PBMC feeder cells. However, after this initial stimulation step, allogeneic feeder cells were substituted for autologous feeder cells and a continuous B-LCL line replaced PBMC as the source of autologous APC. Thus, the system immediately became independent of the donor for successful restimulation and propagation of CTL derived from that donor. Allogeneic feeder cells were kept frozen, further adding to the simplicity and convenience of this system.

Allogeneic feeder cells were not used in the initial stimulation step, because induction of allospecific CTL may have hindered the recovery of antigen-specific CTL. Likewise, use of B-LCL as APC in the initial stimulation step was omitted, because EBV-specific CTL were produced.

Although this report deals with the propagation of CD4+ CTL (which are generally restricted by HLA class II molecules [6, 7, 10, 22]), the system described here may also serve to restimate class I-restricted CD8+ CTL in some cases, for the following reasons. B-LCL readily present antigen in conjunc-

FIG. 2. CTL maintained with allogeneic feeder cells and B-LCL as APC did not exhibit allospecific or EBV-specific reactivity. CTL clones were used as effector cells against autologous B-LCL pulsed with Toxoplasma antigen (T007/Ag) or control fibroblast antigen (T007/HFA) or with the allogeneic, partially major histocompatibility complex class II-matched B-LCL line Sweig pulsed with Toxoplasma antigen (Sweig/Ag). The effector-to-target-cell ratio was 3:1, and the assay was performed when all cloned cell had been in culture approximately 9 months. Maximum ³¹Cr release was 6,632 cpm.

FIG. 3. B-LCL infected with live Toxoplasma tachyzoites served as target cells for autologous CD4+ Toxoplasma-specific CTL. The assay was performed at an effector-to-target-cell ratio of 1:1. Tg, B-LCL infected with live tachyzoites; HFA, B-LCL pulsed with control fibroblast antigen. Maximum ³¹Cr release was 4,288 cpm.
tion with HLA class I molecules, serving as target cells for assessment of cytolysis mediated by class I-restricted CD8+ CTL (4, 13, 16, 20, 24, 29, 33, 34). Furthermore, B-LCL were used as APC for the propagation of class-I restricted CD8+ CTL in previous work (14, 30). Our inability to generate CD8+ Toxoplasma-specific CTL precluded our studying this type of effector cell in this system. This apparent relative inability to present these antigens via the class I pathway, if proven to be correct, is not a property intrinsic to B-LCL but, rather, may relate to defective processing or presentation of antigens of this specific intracellular parasite in the class I pathway (unpublished data).

The reason for the loss of cytolysis in these cells was not clear. CTL were probably clonal (6) and maintained their CD3+ CD4+ phenotype. It may be that the nonclonal B-LCL used in these experiments changed over time in such a way as to hinder CTL propagation. However, the gradual decrease in cell growth and loss of cytolitic activity were similar to those observed when we propagated human CD4+ HIV envelope-specific CTL lines with an anti-T-cell antibody as the stimulus to proliferation (rather than B-LCL) and allogeneic feeder cells (7).

B-LCL have previously been shown to present antigen in the context of class II molecules following infection with recombinant vaccinia viruses encoding single human immunodeficiency virus genes (7, 18, 23, 26) and with other viruses (11, 22, 37). APC may also present class II-restricted peptides following brief incubation with these molecules (15, 23). We now extend these observations to demonstrate that B-LCL may be incubated in the presence of complex protein mixtures and will efficiently take up and process the proteins for effective presentation by the class II pathway. Thus, with no a priori knowledge of immunogenic epitopes, one may elicit and characterize class II-restricted CTL to complex organisms such as unicellular parasites or tumor cells. Furthermore, infection with live Toxoplasma tachyzoites also led to presentation of Toxoplasma antigens in the context of class II molecules.

It has been demonstrated that whole proteins may be cleaved with cyanogen brodime to yield peptide fragments capable of eliciting a CD8+ class I-restricted CTL response, even though these peptides are introduced to APC as exogenous antigen (31, 32). More recently, exogenous proteins were encapsulated into liposomes, allowing efficient introduction into the class I antigen-processing pathway with elicitation of CD8+ CTL (19, 25). We recently described a novel adenovirus system capable of efficient transduction of foreign genes into B-LCL (5). With this broad assortment of techniques (recombinant viruses, chemically cleaved proteins, liposome-encapsulated proteins, receptor-mediated endocytosis pathway reagents, etc.), there may be ample opportunity to use B-LCL to produce class I-presenting APC. Nonetheless, were this system not suitable for propagation of CD8+ CTL, it would still be of use, because the study of CD4+ CTL has become increasingly important (1, 6, 7, 11, 12, 18, 22, 25, 26, 30, 37, 38).

It is likely that optimal conditions for propagation of individual clones, stimulated by B-LCL APC, will have to be defined on a case-by-case basis. We were therefore not rigorous in our study of optimal APC/CTL ratios. However, maximal density appears to be approximately an APC/CTL ratio of 1:4 for the conditions used in these studies. The reasons for growth inhibition of CTL at these high B-LCL densities are not known at present. However, Liu et al. (18a) recently reported that during the course of CTL assays, some B-LCL produced interleukin-6 in concentrations sufficient to inhibit CTL activity. This observation is important from the standpoint of use of B-LCL as APC as well as for their use as CTL target cells and will require further study.

In summary, we present a system for the efficient propagation of human CD4+ CTL. Frozen allogeneic feeder cells replace fresh autologous feeder cells, and long-term B-LCL lines serve as autologous APC. Thus, reliance on a steady donor is greatly diminished. Although our studies focused on CD4+ CTL, the system is probably suitable for propagation of CD8+ CTL in some instances as well, as evidenced by prior published reports. The system may not be suitable for all CTL and was limited, at least in this instance, by the inability to propagate cells for more than 9 months with sufficient cytolytic activity. Other potential concerns relate to the lack of clonality of the B-LCL we used to stimulate cells and the potential for some B-LCL to produce inhibitory cytokines. Nonetheless, given the difficulties in propagating human CTL clones, this method may prove useful to immunologists studying human immunity requiring propagation of CTL in some instances.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Colorado Advanced Technology Institute (to T.J.C.).

Recombinant human interleukin-2 was the generous gift of Hoffman-LaRoche.

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