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EPSTEIN-BARR VIRUS-SPECIFIC T-CELL RECOGNITION OF B-CELL TRANSFORMANTS EXPRESSING DIFFERENT EBNA 2 ANTIGENS

L.E. WALLACE[1], L.S. YOUNG[1], M. ROWE[1], D. ROWE[2] and A.B. RICKINSON[1,3]

[1]*Department of Cancer Studies, University of Birmingham Medical School, Birmingham, B15 2TJ; and* [2]*Ludwig Institute for Cancer Research, St. Mary's Hospital Medical School, Norfolk Place, London W2 1PG, UK.*

Epstein-Barr (EB) virus isolates can be classified as type A or type B depending upon the identity of the virus-encoded nuclear antigen EBNA 2; the EBNA 2A and 2B proteins show limited amino-acid homology and induce largely non-cross-reactive antibody responses in humans. To examine whether EBNA 2 might also be a target for virus-specific cytotoxic T-cell responses (like "intracellular" antigens in other viral systems), normal B cells from non-immune donors of known HLA type were transformed *in vitro* with virus isolates either of type A (from the B95-8 and IARC-BL74 cell lines) or of type B (from the AG876 and IARC-BL16 cell lines) to provide a suitable panel of target cells. DNA hybridization with type-specific probes and immunoblotting with type-specific antisera confirmed the EBNA 2 type of the resident virus in the various *in vitro* transformants. These cells were then tested as targets for virus-specific cytotoxic T cells, the latter being prepared from type-A virus-infected donors by *in vitro* reactivation of memory cells from peripheral blood using autologous type-A virus-transformed cells as stimulators. Such effector cells lysed type-A virus-transformed and type-B virus-transformed target cells equally well, indicating that EBNA 2 (in particular that part of the protein which varies between virus types) seems not to be a dominant antigen for the induction of EB virus-specific cytotoxic responses.

Epstein-Barr virus (EBV) transforms human B lymphocytes *in vitro* into permanent B-lymphoblastoid cell lines in which every cell retains multiple episomal copies of the viral genome. Analysis of viral gene expression in such lines to date has identified 4 viral antigens which appear to be constitutively expressed in the transformed cells; these consist of 3 distinct nuclear antigens, EBNA 1 encoded within the Bam HI K fragment of the viral genome (Strnad *et al.*, 1981; Summers *et al.*, 1982), EBNA 2 encoded within the Bam HI WYH region (Dambaugh *et al.*, 1984; Hennessy and Kieff, 1985), and EBNA 3 largely encoded within the Bam HI E fragment (Hennessy *et al.*, 1986), as well as a latent membrane protein (LMP) encoded within the Bam HI N fragment (Fennewald *et al.*, 1984; Hennessy *et al.*, 1984). All 4 antigens have been identified by serological means, using naturally-occurring human sera in the case of the various EBNA proteins and rabbit antisera to a relevant hybrid fusion protein in the case of LMP. Other more recently reported EBV latent gene products may exist but have yet to be properly characterized (Kallin *et al.*, 1986; Sample *et al.*, 1986).

The presence within all previously-infected individuals of memory T lymphocytes (cytotoxic precursors) with specificity for autologous EBV-transformed B cells (Moss *et al.*, 1978, 1981) indicates that one or more of the viral antigens associated with the transformed state is also immunogenic to the T-cell system. At the moment, the target antigens for such EBV-specific cytotoxic T-cell responses are known collectively as the "lymphocyte-detected membrane antigen" (LYDMA), but this is purely an operational definition which will be redundant once the true identity of the virus-induced target structures has been resolved (Rickinson *et al.*, 1981). In this regard, all 4 serologically-defined antigens (EBNAs 1, 2 and 3, and LMP) could conceivably serve to elicit T-cell cytotoxicity. Certainly, one can no longer argue that LMP, because of its association with plasma membranes, represents the most likely candidate for LYDMA; work in several viral systems, in particular recent studies with influenza virus, has made it clear that antigens which by serological criteria appear to be exclusively intracellular are nonetheless represented on the cell membrane in a form which can be recognized by cytotoxic T cells (Townsend *et al.*, 1985). Accordingly, a possible role for the EBNA proteins as T-cell-detected target antigens must be seriously borne in mind. Our particular interest in EBNA 2 stemmed from the results of recent experiments in which EBV-positive Burkitt lymphoma cell lines, known to be operationally LYDMA-negative when tested as targets in cytotoxicity assays (Rooney *et al.*, 1985), revealed an unusually restricted pattern of EBV latent gene products with both EBNA 2 and LMP often not detectably expressed (Rowe *et al.*, 1986).

The present experiments were initiated with the specific aim of looking for EBNA-2-directed T-cell responses by taking advantage of the existence of two types of EBV isolate encoding immunologically distinct EBNA 2 proteins. Thus, all Western isolates of EBV so far examined show strong homology with the prototype B95-8 and M-ABA virus strains in their EBNA 2-encoding region, and synthesize a very similar 85 kDa EBNA 2 "type-A" protein. In contrast, some EBV isolates from equatorial regions of Africa (of which the Jijoye and AG876 virus strains are prototypes) show only weak genome homology with B95/M-ABA virus in this region, and encode a smaller 75 kDA EBNA 2 "type B" protein (Dambaugh *et al.*, 1984; Adldinger *et al.*, 1985; Zimber *et al.*, 1986). Naturally-occurring antibodies against EBNA 2A show very little cross reactivity with EBNA 2B, and vice-versa, so that the two proteins appear to be serologically distinct. Any cytotoxic T-cell responses mediated through recognition of a membrane-associated form of EBNA 2 might therefore be expected to show a similar EBNA-2 type preference. The present experiments were designed to examine this particular point.

MATERIAL AND METHODS

Culture Medium

All cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% foetal calf serum.

Cell lines

The B95-8 (Miller and Lipman, 1973) and AG876 (Pizzo *et al.*, 1978) cell lines were used as the prototype sources of EBNA 2A- and EBNA 2B- positive virus isolates, respectively. In addition the IARC-BL 74 and IARC-BL 16 cell lines (Lenoir *et al.*, 1985) were obtained from Dr. G. Lenoir, (IARC Laboratories, Lyon, France) as an alternative source of type-A (BL74) and type-B (BL16) virus isolates (Zimber *et al.*, 1986). Control cell lines used in EBNA-2 typing experi-

[3]To whom reprint requests should be sent.

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ments included the X50-7 (type-A) and JC-5 (type-B) reference lines (Wilson and Miller, 1979; Rowe *et al.*, 1986), the EB-virus-positive cell line P_3 HR_1 in which the resident virus has a deletion of the entire EBNA 2-coding region (Adldinger *et al.*, 1985) and the EB-virus-negative lines Louckes (van Santen *et al.*, 1981), EB4 (Wallace *et al.*, 1982*a*) and Ramos (Klein *et al.*, 1975).

Target cell lines for cytotoxicity assays were produced by co-cultivation of peripheral blood mononuclear cells from EBV antibody-negative (seronegative) healthy adult donors, of known HLA antigen type, with X-irradiated cells of the above virus-producer cell lines. Co-cultures were set up in the presence of Cyclosporin A (CSA) at a concentration of 0.1 μg/ml culture medium, in order to avert any co-incidental T-cell activation *in vitro* (Rickinson *et al.*, 1984), and thereafter were re-fed weekly with a half change of medium (CSA-free) until foci of virus-transformed B cells could be transferred out of the co-culture wells and used to establish a permanent cell line.

DNA hybridization for EBNA-2 typing

Cellular DNA was isolated by SDS-proteinase K lysis followed by repeated phenol and chloroform-isoamyl alcohol extraction and precipitation with ethanol. Ten micrograms of DNA were digested with 20 U Bam HI for 5 hr and the DNA fragments were separated in horizontal 0.7% agarose gels run at 30 v for 18 hr. The separated DNA fragments were transferred to nitrocellulose as described by Southern (1975).

The construction of the cloned probes pM-Bam H2 and pJ-HKA7 used to identify EBNA 2A and 2B, respectively, has been described (Polack *et al.*, 1984; Adldinger *et al.*, 1985); these probes were kindly supplied by Dr. G. Bornkamm, Institut fur Virologie, Freiburg; pM-Bam H2 contains the Bam H2 fragment of M-ABA virus (type A) in pACYC184, and pJ-HKA7 contains the Hinc II-Pst I fragment of Jijoye virus (type B) in pUC8. The cloned probes were labelled with biotin-11-dUTP (BRL) by nick translation. Hybridization of heat-denatured biotin-labelled DNA was carried out in 45% formamide, 10% dextran sulphate and 6 × SSC in the presence of heat-denatured salmon sperm DNA (250 μg/ml) as carrier. The hybridized probes were detected by indirect immunocytochemistry. Filters were incubated with rabbit anti-biotin IgG (Enzo Biochem, New York, NY), washed and then incubated with a biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA). After a subsequent incubation with avidin DH-biotinylated horseradish peroxidase complex (Vector ABC kit) the filters were developed in diaminobenzidine tetrahydrachloride and hydrogen peroxide.

SDS-polyacrylamide gel electrophoresis and Western immunoblotting for EBNA-2 typing

The cells were solubilized in sample buffer (0.625M Tris, pH 6.8, 2% SDS, 0.1% 2-mercaptoethanol, 10% glycerol and 0.02 mM bromophenol blue) and proteins separated by the discontinuous gel electrophoresis technique of Laemmli (1970). Gels, 1.5 mm thick and 180 mm long, consisted of 10% resolving gels (acrylamide:bis-acrylamide = 30:0.8) and a 5% stacking gel. Generally, 30 μl of sample representing 2 to 3 times 10^6 cells were electrophoresed at 60 v for 12 hr and blotted on to nitrocellulose (Towbin *et al.*, 1979; Fischer *et al.*, 1984). After blotting, the non-specific antibody binding sites on the filter were blocked with dilute skim milk according to the method of Johnson *et al.* (1984). Molecular weight determinations were made using pre-stained high- and low-molecular-weight standards (BioRad, Richmond, CA), run on the same gels.

Preparation of EBV-specific T cells

EBV-specific cytotoxic T-cell preparations were obtained from selected virus-immune donors by co-cultivating peripheral blood mononuclear cells with low numbers of X-irradiated stimulator cells from the autologous B95-8 virus-transformed B-cell line, exactly as previously described (Wallace *et al.*, 1982*a*). Effector T cells prepared in this way were expanded as interleukin 2 (IL2)-dependent T-cell lines using IL2-conditioned medium and regular re-exposure to the relevant X-irradiated stimulator cells (Wallace *et al.*, 1982c).

Preparation of allo-reactive T cells

Allo-reactive cytotoxic T cells with specificity for the HLA-A2.1 ("common") antigen were prepared by co-cultivating peripheral blood mononuclear cells from a virus-non-immune donor RT (HLA-type A2.2, A3; B7, B57) with low numbers of X-irradiated stimulator cells from the allogeneic B-cell line from donor JY (HLA-type A2.1, A2.1; B7, B7) and expanding the reactive cells in IL2-conditioned medium as above; the allo-specificity of these cells is fully documented elsewhere (Wallace *et al.*, 1985). In the present report, reference to HLA-A2 subtypes is not necessary; all the A2-positive individuals used (with the exception of RT) in fact possessed the "common A2" antigen.

Cytotoxicity assays

Effector T cells from the IL2-dependent T-cell lines were cultured in normal medium (IL2-free) overnight to remove any residual surface-bound lectin before use in the assay. Chromium release assays were conducted over 5 hr at effector: target ratios of between 2.5:1 and 10:1 (Moss *et al.*, 1981). In one set of experiments, target cells were pre-incubated with high concentrations of the monoclonal antibody W6/32 (Barnstable *et al.*, 1978), specific for a monomorphic determinant on all HLA class-I antigens, for 20 min at 37°C in the assay well before addition of the effector cells (Wallace *et al.*, 1981). The antibody was used at a final concentration of 1/600 of an ascitic fluid preparation (Sera-Lab, Crawley Down, UK).

RESULTS

Identification of EBNA 2 type of target cells

Sets of EB-virus-transformed B-lymphoblastoid lines were obtained from each of two seronegative adult donors (CD and CR) for the purpose of these experiments; each set consisted of 4 lines in which the source of the transforming virus was X-irradiated cells of either the B95-8, BL74, AG876 or BL 16 cell line.

Figure 1 shows representative results obtained when BamHI-digested DNA from the transformed lines, and from each of the original virus-producing lines, was probed with the M-ABA-derived (*a*) and with the Jijoye-derived (*b*) cloned sequences. The B95-8 and BL74 cell lines, and the normal B-cell lines transformed by these viruses, gave a BamHI fragment of about 6.9 Kb which hybridized preferentially to the M-ABA-derived probe (*a*). In contrast, the AG876 and BL16 cell lines, and their corresponding normal B cell transformants, gave a fragment of about 8.5 Kb which hybridized preferentially to the Jijoye-derived probe (*b*). The cross-hybridization to the non-preferred probe which was observed under these conditions was specific and reflected the partial homology which exists between the two cloned sequences; DNA from the EB-virus-negative control cell lines EB4 and Ramos, and from the EBNA 2 deletion mutant line P_3HR_1, gave no detectable hybridization with either probe (data not shown).

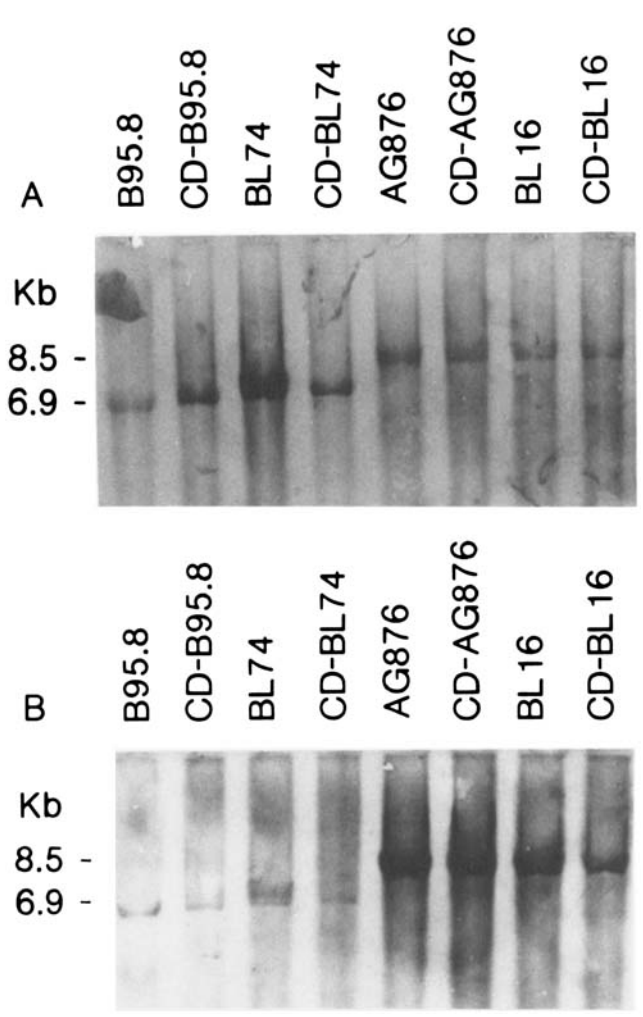

FIGURE 1 – Hybridization of biotin-labelled M-ABA virus-derived (*a*) and of Jijoye virus-derived (*b*) probes to filters containing separated BamHI DNA fragments of the cell lines indicated. Each lane received 10 μg DNA.

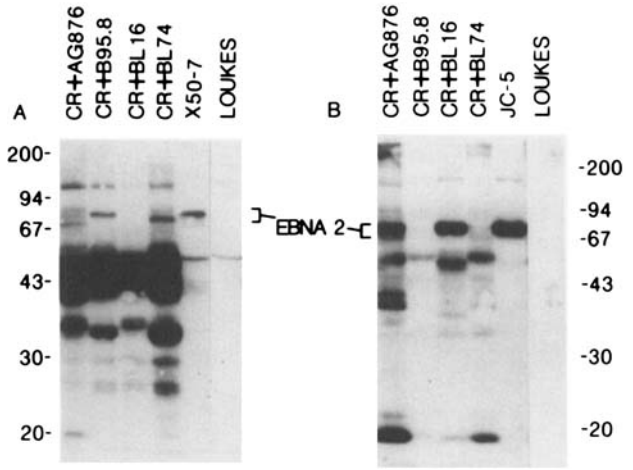

FIGURE 2 – Western immunoblot detection of the EBNA 2 proteins in the lymphoblastoid cell lines established from donor CR by transformation with AG876, B95-8, BL16- and BL74-derived virus. After separation of cellular proteins on polyacrylamide gels and blotting on to nitrocellulose, the filters were probed with a 1:100 dilution of (*a*) WL, anti-EBNA-2A-positive, anti-EBNA-1-negative, anti-EA-positive serum, or (*b*) AM anti-EBNA-2B-positive, anti-EBNA-1-negative, anti-EA-positive serum. Antibody binding proteins were detected with ^{125}I-labelled staphylococcal protein A. X50-7 and JC-5 are reference EBNA 2A- and EBNA 2B-positive cell lines, respectively, and LOUKES is an EBV-negative lymphoma cell line included as a control.

The DNA hybridization results, which indicated that the normal B-cell transformants do indeed carry the expected virus type, were further supprorted by immunoblotting data. Thus, using serum WL with significant anti-EBNA 2A antibody reactivity, we detected EBNA 2A expression in the B95-8- and BL74-transformants in the same way as in the type-A reference cell line X50-7 (Fig. 2a); there was possibly some cross-reactivity with the EBNA 2 protein in the AG876-transformant. However, when serum AM with a particularly strong anti-EBNA 2B reactivity was used, EBNA 2B was easily detectable in the AG876- and BL16-transformants and in the type-B reference cell line, JC-5 (Fig. 2*b*). The differential blotting patterns observed with these sera in the 75-85 kDa region of the gel not only identify unequivocally the type of EBNA 2 protein being expressed in the various transformed lines, but also emphasize the substantial antigenic differences between EBNA 2A and EBNA 2B as perceived by the antibody response in humans.

Cytotoxic T-cell recognition of target cells

EB virus-specific cytotoxic T cells, raised from virus-immune donors by *in vitro* stimulation of blood mononuclear cells with the autologous B95-8-virus-transformed B-cell line, were clearly able to recognize and kill HLA class-I antigen-matched target cells transformed either by EBNA 2A-encoding or by EBNA 2B-encoding virus isolates. The relevant data are illustrated in Figures 3 and 4; these are representative of results seen on repeated occasions of testing with the two sets of target lines described.

Thus, the various target lines derived from seronegative donor CD were all significantly lysed by effector T cells from the HLA-matched virus-immune donors DG (Fig. 3*a*) and JG (Fig. 3*b*). In this particular case, the B95-8-virus-transformed target line sustained slightly higher lysis than the corresponding lines transformed by the BL 74, AG876 and BL 16 virus isolates. Clearly, the levels of killing observed in these assays were all well above the background values obtained when, as a control, these same targets were exposed to virus-specific effector T cells from an HLA-mismatched donor CF (Fig. 3*c*).

The particular effector populations used in Figure 3*a* and *b* were chosen because both contained a demonstrable HLA-A2 antigen-restricted component of cytotoxicity which was responsible for recognition of the CD target lines. Thus, effector donor DG shared no HLA class-I antigens other than HLA-A2 with CD, whilst effector donor JG shared both HLA-A1 and -A2 but mounted no HLA-A1-restricted T-cell response (Gaston *et al.*, 1985). Given that the virus-specific recognition of CD targets was HLA-A2-restricted, the additional results, shown in Figure 3*d*, provide a particularly relevant control. Here allo-specific T cells, whose cytotoxicity was directed entirely towards HLA-A2 as an allo-antigen, were tested against the same panel of CD target lines; once again, all the

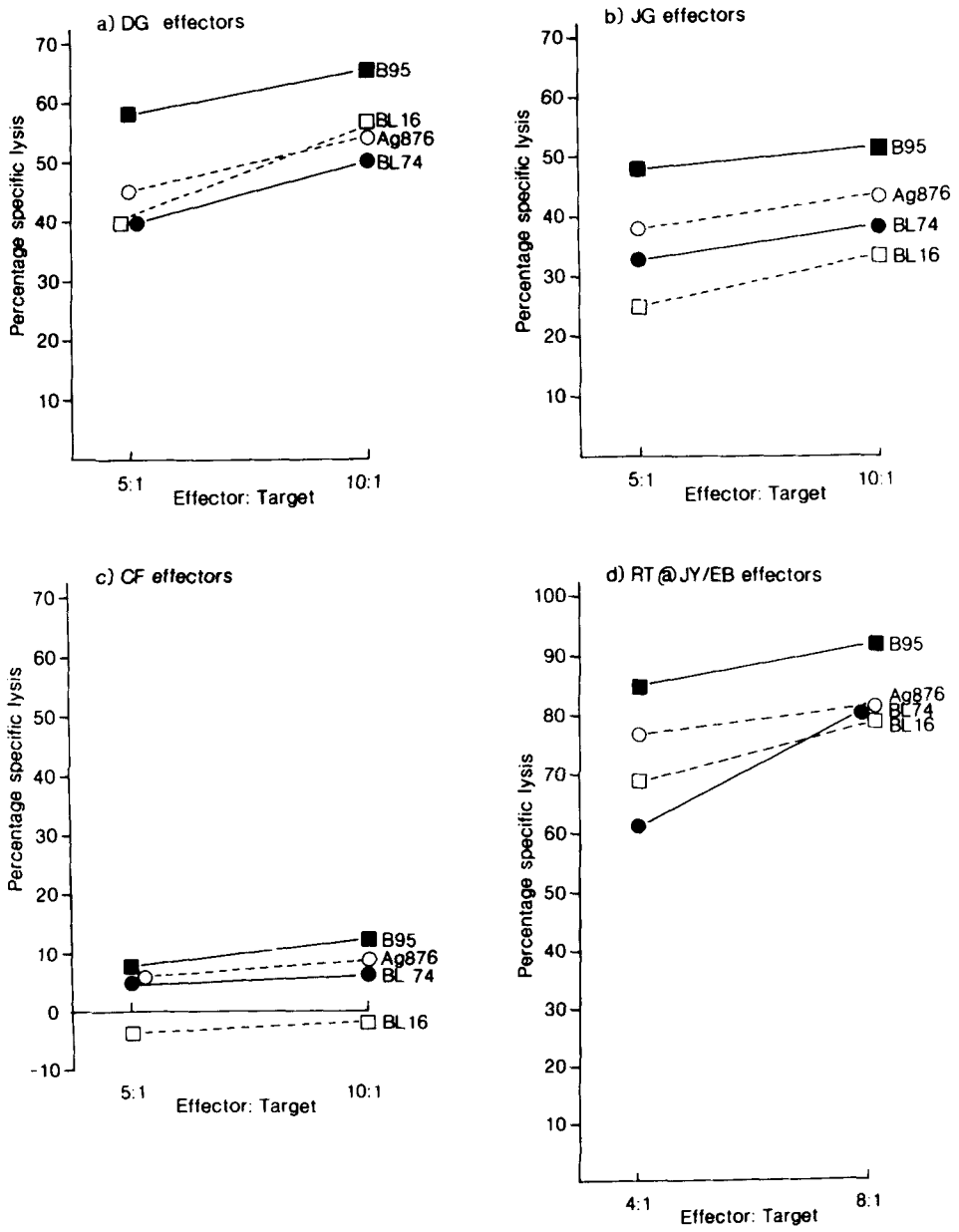

FIGURE 3 – Susceptibility of target B cells from donor CD (HLA-A1, A2; B37, B62) transformed by virus from B95.8 cells (■), BL 74 cells (●), BL 16 cells (□) or AG876 cells (○), to lysis by EB-virus-specific, HLA-matched effector T cells from donors DG (*a*) and JG (*b*), EB virus-specific, HLA-mismatched effector T cells from donor CF (*c*) and by anti-HLA-A2 allospecific effector T cells from donor RT (*d*). Results of one representative experiment are expressed as percentage specific lysis of target cells at two different effector:target ratios. DG effectors are matched with the CD target cells through HLA-A2; and JG effectors (*b*) are matched through HLA-A1 and -A2.

targets sustained high levels of lysis, with the B95-8 virus-transformed line giving the highest values.

A second set of target cell lines, derived from seronegative donor CR, gave an essentially similar pattern of results which was once again observed in repeated asays. Figure 4 shows that 3 different effector T-cell populations, recognizing CR targets either through shared HLA-A2 and -B8 antigens (DG effectors, Fig. 4*a*; JG effectors, Fig. 4*b*) or through shared HLA-A2 and B7 antigens (CE effectors, Fig. 4*c*), mediated significant lysis of CR target lines whether they carried EBNA 2A - encoding (B95-8, BL 74) or EBNA 2B-encoding (AG876, BL 16) virus isolates. Generally, all 4 target lines showed comparable levels of lysis, just as was observed when these same targets were tested for their sensitivity to HLA-A2-directed allospecific effectors (Fig. 4*d*). It was interesting, in the course of these experiments, to note the occasional exception to this pattern. Thus, as shown in Figure 4*a*, DG effector cells appeared to lyse B95-8 and BL 74-virus transformants

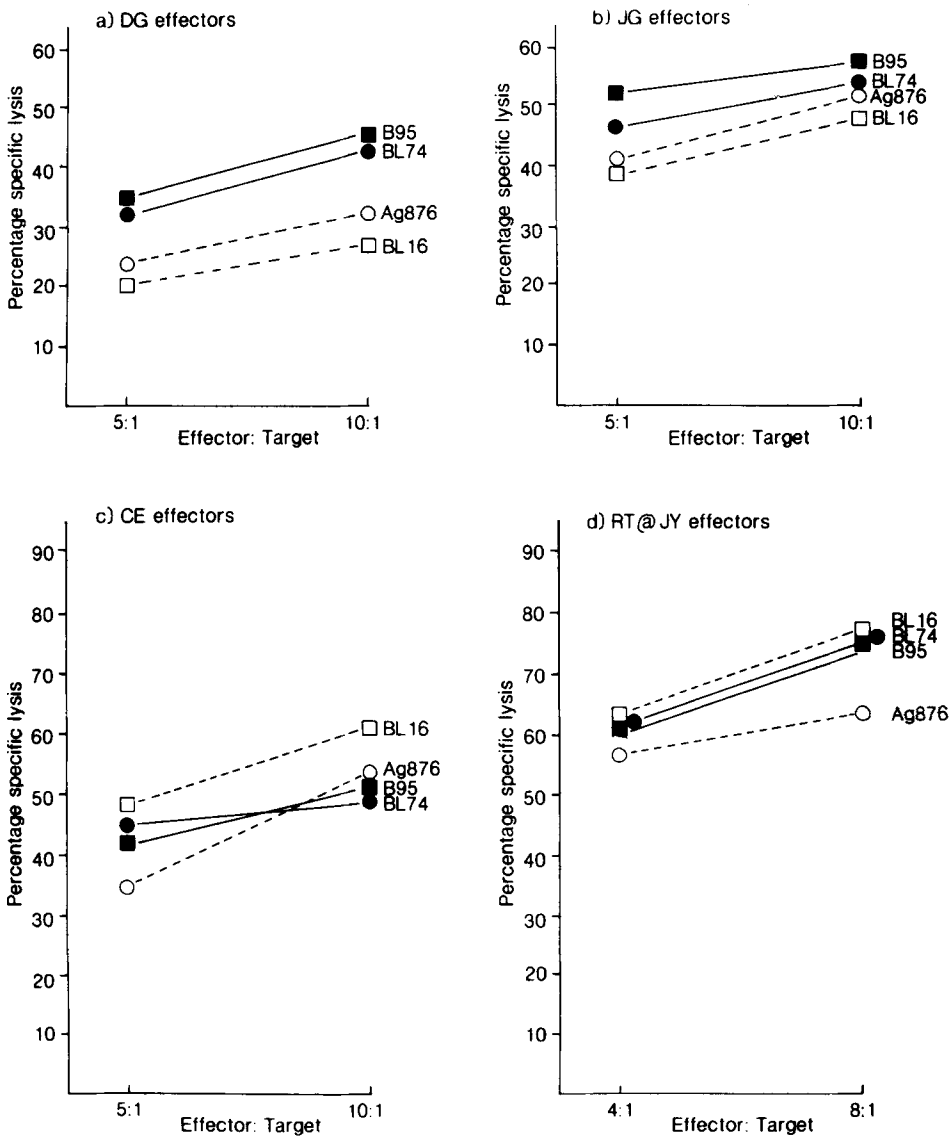

FIGURE 4 – Susceptibility of target B cells from donor CR (HLA-A2, A11; B7, B8) transformed by virus from B95.8 cells (■), BL 74 cells (●), BL 16 cells (□) or AG 876 cells (○), to lysis by EB-virus-specific, HLA-matched effector T cells from donor DG (*a*), JG (*b*) and CE (*c*), and by anti-HLA-A2 allospecific effector T cells from donor RT (*d*). Results of one representative experiment are expressed as percentage specific lysis of target cells at two different effector:target ratios. DG and JG effectors are both matched with the CR target cells through HLA-A2 and -B8, and CE effectors are matched through HLA-A2 and -B7.

more readily than the corresponding AG876- and BL 16-virus transformants, a result noted on several occasions of testing these particular effector-target combinations.

Finally, monoclonal antibody (MAb)-blocking studies confirmed that the virus-specific effector populations used in these experiments were indeed recognizing the relevant target lines in a specific HLA class-I-restricted manner. This is illustrated by an experiment (Fig. 5) in which effector T cells from another virus-immune donor (St G) were tested against the autologous B95-8 virus-transformed target cell line and against the set of HLA A2, B8-matched CR target cell lines, with and without pre-exposure of the targets to saturating concentrations of the W6/32 MAb specific for a framework determinant on all HLA class-I antigens. In every case, there was substantial blocking of T-cell recognition of the HLA class-I matched targets; moreover, in the same assay, HLA class-I-mismatched target lines showed minimal levels of lysis irrespective of the presence or absence of the MAb.

DISCUSSION

By using cell lines of known EBNA 2 type as a source of transforming virus, it has been possible to establish sets of B lymphoblastoid lines derived from the same donor of origin but transformed by different virus isolates. These lines, used as target cells in the present experiments, not only display the EBNA-2 type of the parental virus by DNA hybridization (Fig.

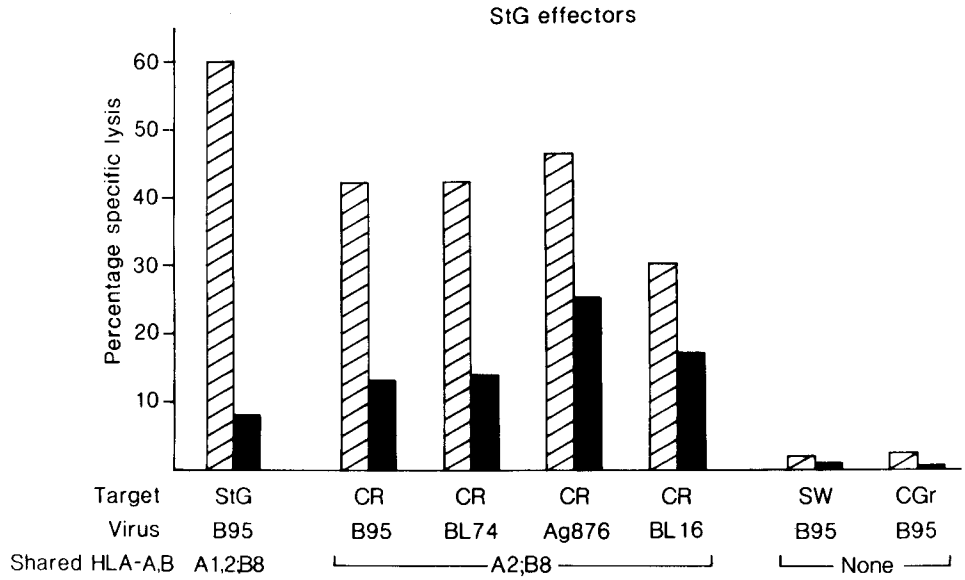

FIGURE 5 – The effect of saturating concentrations of the HLA-A, B, C antigen-specific MAb W6/32 upon the level of lysis of target cells from donor CR (HLA A2, A11; B7, B8) transformed with EB virus from B95, BL 74, AG876 or BL16 cells, by EB-virus-specific effector T cells from donor StG. Results of one representative experiment are expressed as percentage specific lysis of target cells at an effector:target ratio of 10:1 in the presence (■) or absence (▨) of monoclonal antibody. The effect of antibody on the level of lysis of autologous target cells (StG) and of HLA-mismatched targets (SW, CGr) all transformed with EB virus from B95.8 cells, is included for comparison. The degree of HLA-A and B antigen sharing with the effectors is shown below each target.

1), but clearly express the relevant EBNA 2A or 2B protein at easily detectable levels in Western immunoblots (Fig. 2). Sequence comparison of the B95-8 and AG876 virus genomes in the EBNA-2-encoding region indicates that EBNA 2A and 2B share just over 50% homology at the amino-acid level. It is therefore not surprising that the two proteins induce antibody responses in humans which are largely non-cross-reactive (Dambaugh *et al.*, 1984; Dillner *et al.*, 1985*a* and Fig. 2) and that antisera to EBNA-2A-derived fusion proteins and synthetic peptides show a similar type-specific reactivity (Dillner *et al.*, 1985*b*; Hennessy and Kieff, 1985; E. Kieff, personal communication).

Since T-cell responses are likely to be just as sensitive to sequence variation of this kind, any cellular reactivity to EBNA 2 might also be expected to show type-specific preference. In designing the present experiments it was important to bear in mind recent virological (Zimber *et al.*, 1986) and seroepidemiological (Sculley *et al.*, 1984; Dillner *et al.*, 1985*a*) evidence which indicates that Western societies, from which all our effector cell donors were drawn, are infected almost exclusively with "type A" isolates of EBV. Accordingly, it was necessary to generate the effector-cell preparations used here by co-cultivating memory T cells from these donors with B95-8 virus-transformed autologous B cells, so that both *in vivo* priming and *in vitro* boosting of the EBV-specific response had involved "type A" virus isolates.

The results from the main body of experiments are clear-cut. Effector T cells generated in this way recognize EBNA 2A-positive and EBNA 2B-positive transformants with equal efficiency (Figs. 3, 4 and 5). Only rarely was there evidence for any preferential lysis of EBNA 2A-positive transformants (Fig. 4*a*). The overall pattern of results suggests either: (i) that the EBNA 2 protein is not a dominant antigen for the induction of EBV-specific T-cell cytotoxicity; or (ii) that if it is immunogenic to T cells, then reactivity is directed towards the extreme N-terminal region of the molecule which is the only part well conserved in both "type A" and "type B" proteins (Dambaugh *et al.*, 1984).

Once again it must be stressed in interpreting these results that the cytotoxic T-cell response to EBV is polyclonal. Individual components within effector T-cell lines are known to be restricted through different HLA class-I antigens (Wallace *et al.*, 1982*b*) and there is no *a priori* reason why the viral antigen specificity of these components should not also be just as diverse. For this reason, the somewhat atypical results obtained with DG effector T cells in these experiments should not be ignored (Figs. 3*a* and 4*a*); these effectors recognized all the CD target cell lines (HLA-A2 matched) with equal efficiency whilst showing some preference for EBNA-2A-positive targets within the CR set of lines (HLA A2, B8 matched). Whilst there may be some trivial explanation for these differences, it is nevertheless possible that DG effector cells contain a B8-restricted component with specificity for the EBNA 2A protein. Further work will be required to examine this possibility more carefully.

From the overall pattern of results presented here, however, it is clear that other experimental approaches will be required to identify unambiguously those viral antigens which are the preferred targets for EBV-specific cytotoxic T-cell responses. Such approaches might exploit the existence of EBNA 2-deletion mutants of EBV (such as the P_3HR_1 strain) or the availability of DNA constructs which have already achieved expression of the EBNA 2 gene after transfection into rodent cells (Dambaugh *et al.*, 1986).

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