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Specific lysis of Varicella Zoster Virus-Infected B Lymphoblasts by Human T Cells

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Epstein-Barr virus-transformed human B cells expressed cell surface varicella-zoster virus (VZV) antigens after superinfection with VZV although they did not form infectious centers in a plaque assay. The VZV-superinfected cells were lysed by autologous VZV-stimulated T-cell lines and their derivative clones. The effector cells were specific for VZV and an HLA DR antigen and were $T4^+$. The specificity of lysis of Epstein-Barr virus-transformed, VZV-superinfected targets by prestimulated mononuclear cells in this system contrasted with the unrestricted lysis seen when the targets were VZV-infected fibroblasts.

Infection with varicella-zoster virus (VZV) elicits the production of antibody and of sensitized T lymphocytes which proliferate in VZV antigen-stimulated cultures (18). Attention has recently focused on the VZV antigens which are recognized by the immune system (16) and particularly by T cells, after the appreciation of a temporal relationship between waning in vitro T-cell responses to VZV in the elderly and their increased susceptibility to herpes zoster (1, 13). Both glycoprotein and nucleocapsid antigens elicit proliferative responses by T cells from immune donors, but little is known of the induction of T-cell cytotoxicity. We previously showed that VZV-infected fibroblasts were lysed by natural killer cells with the Leu-11^+ phenotype (A. R. Hayward, M. Herberger, and M. Laszlo, Clin. Exp. Immunol., in press) and that the rate of target cell lysis was increased by lymphokines derived from T cells. However, VZV-infected fibroblasts do not express class II major histocompatibility complex (MHC) antigens; therefore, they are not suitable targets for investigating the cytotoxic potential of the $T4^+$ cells which account for most of the in vitro proliferative response (D. Suez and A. Hayward, Fed. Proc. **44**:1312, 1985). We now report that Epstein-Barr virus (EBV)-transformed B-cell lines (which have class II MHC antigens) may be superinfected with VZV and then used as targets for cytotoxicity by VZV-stimulated T lymphoblasts.

MATERIALS AND METHODS

Blood MNC and EBV line preparation. Blood mononuclear cells (MNC) were separated from defibrinated blood by Ficoll-Hypaque centrifugation. They were washed twice in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered Hanks balanced salt solution and suspended in RPMI 1640. MNC to be transformed with EBV were first depleted of T cells by rosetting with sheep erythrocytes treated with 2-aminoethyliosthiuronium (15). EBV was prepared from 10^7 B95-8 cells (14) by two freeze-thaw cycles and sonication. The debris was centrifuged at 800 × *g* for 10 min, and the supernatant was filtered (pore size, 0.45 μm), diluted 1:15, added to the non-T cells, and cultured in RPMI 1640 containing 10% fetal calf serum and 10^{-5} M 2-mercaptoethanol until colony growth was detectable. EBV-transformed lines were expanded in the same medium in tissue culture flasks. Donors and EBV B-cell lines were typed with NIH HLA-DR trays.

Preparation of VZV-specific T-cell clones. Blood MNC (10^6) were cultured in RPMI 1640 with 10% autologous serum and 10^5 glutaraldehyde-fixed VZV-infected HLA-matched fibroblasts (prepared as described in reference 2). After 7 days, the cultures were diluted to twice their original volume in medium with 10 U of recombinant IL-2 (catalog no. 01010; Amgen, Thousand Oaks, Calif.). After a further week, blast cells were counted on a Coulter Counter with the threshold setting adjusted to exclude small lymphocytes. The cells were then suspended in medium with a 1:100 dilution of VZV antigen (18) to a cell concentration of 10^5 blasts per ml. Autologous blood MNC, irradiated with 2,000 rads, were added to a final concentration of 10^6/ml. After 4 days, the cultures were diluted to 10^5 blast cells per ml in fresh medium containing 10 U of IL-2 per ml and incubated for 3 days before being returned to medium containing antigen plus irradiated cells. For cloning, the T-cell blast cells were enriched on a Percoll gradient (11) and plated with a 1:100 dilution of VZV antigen (18) on irradiated autologous MNC at 100, 10, and 1 cell per well with 1 U of recombinant IL-2 per ml in Linbro (76-032-05) plates. Wells with visible growth were harvested after 14 days and expanded in 24-well plates on autologous antigen-presenting cells with VZV antigen and IL-2 for a further 10 days. Clones were subsequently maintained by exposure once a week to VZV antigen on antigen-presenting cells and reculturing 3 to 4 days later in medium containing IL-2. Thymidine uptake by these clones was measured in the presence of irradiated autologous MNC as antigen-presenting cells, as described previously (11). Clones were recloned by plating in Linbro plates at 1 cell per well in medium with 1 U of IL-2 per ml and 5 × 10^3 irradiated (5,000 rads), VZV-infected autologous EBV-transformed cells from the B-cell line.

Cytotoxicity assays. EBV-transformed B-cell lines were infected with VZV (Cp5, 262 strain), by adding frozen-thawed VZV-infected HELF cells prepared as previously described (2) to give a multiplicity of infection of 0.5 to 1. Cells were infected with herpes simplex virus (HSV) (Kos strain) or cytomegalovirus (CMV; AD 169 strain) by adding cell-free virus at a multiplicity of infection of 0.5 to 1. VZV-infected and uninfected fibroblasts from HLA-typed donors were used as targets in some experiments. Their

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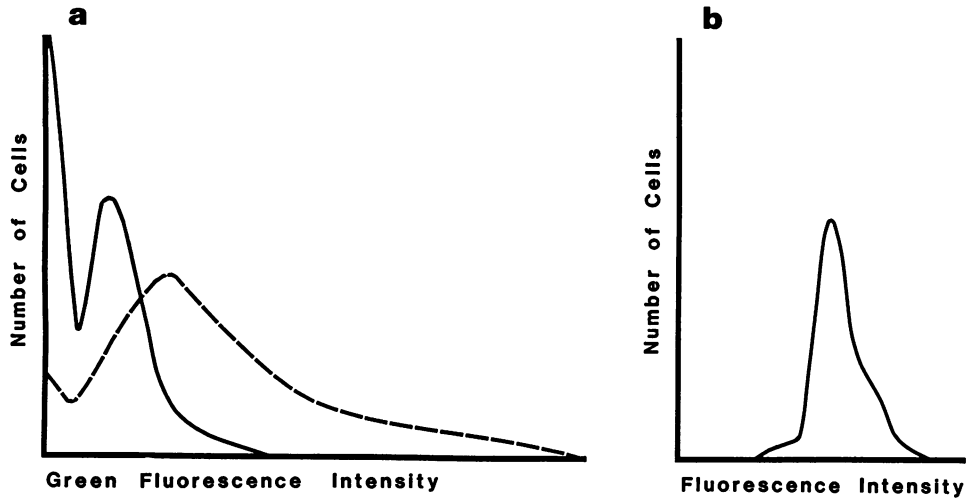

FIG. 1. (a) Fluorescence profiles of EBV-transformed B-cell lines superinfected with VZV and stained with VZV antibody-positive (- - - -) or antibody-negative (——) human IgG and fluorescein-labeled anti-IgG. (b) Profiles of the same line stained for DNA with acridine orange. Two thousand cells were counted.

culture, preparation, and conditions for infection are fully described in reference 2.

Infected or uninfected target cells (1×10^6 to 2×10^6) were labeled with 100 μl of ^{51}Cr (CJS 4V sodium chromate; Amersham Corp., Arlington Heights, Ill.) for 40 min at 37°C and then washed four times and adjusted to 2.5×10^4/ml. For cold target inhibition studies, these targets were suspended in medium already containing a 10-fold excess of the appropriate cold target. For antibody inhibition studies, 10 μl of monoclonal anti-HLA A/B (culture supernatant of ATCC HB95), anti-DQ (final dilution, 1:50; Leu 10; Becton Dickinson and Co., Paramus, N.J.) or anti-DR (final dilution, 1:2,000; catalog no. 7360; Becton Dickinson) were added directly to the culture wells. Targets (0.1-ml portions) were mixed with effectors (0.1-ml portions) in Linbro (76-015-05) plates and incubated for 4 h at 37°C. The plates were then centrifuged ($200 \times g$ for 7 min), and 0.1 ml of the supernatant was aspirated and counted. Percent specific lysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Maximum release was determined by adding 10 μl of lysing agent (Scientific Products, Inc., Detroit, Mich.) to the well.

Characterization of VZV-infected target cells. Rhodamine-conjugated anti-VZV was prepared by conjugating zoster immunoglobulin (lot no. MVZIG-17; American Red Cross) to tetramethylrhodamine isothiocyanate (8). The conjugate $E_1515/280$ was 1.2, and it was used at 0.05 mg/ml. Indirect immunofluorescence was done with VZV antibody-negative normal human serum, zoster immunoglobulin, CMV immunoglobulin diluted to immunoglobulin G (IgG) concentrations in serum (10 mg/ml), and the anti-DR and anti-DQ antibodies, as described in the instructions of the manufacturers. Cells were incubated with 20 μl of antibody on ice for 30 min, washed three times in Hanks balanced salt solution, labeled with fluorescein-conjugated goat anti-human IgG (Wellcome Diagnostics, Research Triangle Park, N.C.) or anti-mouse IgG (Cooper Biomedical, Inc., West Chester, Pa.) washed three times and analyzed by microscopy or on a Coulter EPICS C with an immunoanalysis program. DNA analysis of the cells was done by the method of Darzynkiewicz et al. (5), with acridine orange staining and cytofluorograph analysis for green (DNA) fluorescence.

Infectious center assay. EBV-transformed B cells (1.2×10^6) were incubated with VZV at a multiplicity of infection of 0.5 for 2 h and then washed three times. The cells were cultured in Dulbecco minimal essential medium plus 2% fetal calf serum for 1, 2, 3, or 7 days, at which times samples were tested in 10-fold dilutions for VZV in an infectious center assay on HELF monolayers. Monolayers were fixed on day 7, stained with crystal violet, and examined for VZV plaques.

RESULTS

Characteristics of superinfected target cells. The EBV-transformed B cells were stained with rhodamine-conjugated zoster immunoglobulin on successive days after addition of VZV. The percentage of cells with surface fluorescence was 10% on day 1, 42% on day 2, and 90% on day 3. To exclude Fc receptor binding as a cause for positive staining, the fluorescence profiles of VZV-infected and uninfected cells after staining with VZV antibody-positive and -negative IgG were compared. No staining of uninfected cells was detected, while 83% of the infected cells were positive after staining with the VZV, but not nonimmune, antibody (Fig. 1a). VZV-infected and uninfected B-line cells were both stained with the anti-DR and anti-DQ monoclonal antibodies, and they incorporated similar amounts of tritiated thymidine (4,380 ± 620 cpm for infected cells versus 5,450 ± 870 cpm in a 4-h pulse for uninfected cells; three cell lines were tested). This result suggests that proliferation of the B-cell line was not interfered with as a result of the VZV superinfection. This view is supported by the identical results from DNA analysis of VZV-infected (Fig. 1b) and uninfected cells. This profile also indicates that substantial numbers of cells were in S phase. To determine whether the target cells were indeed permissive for VZV, 10^6 B-cell line cells were infected with VZV and assayed in an infectious center assay 1, 2, 3, and 7 days later. Twenty-five plaques per 10^4 cells were obtained on day 1, but no virus was recovered on the subsequent days. These results indicate that VZV antigens were expressed on the surface of the EBV-transformed B cells, but the lack of infectious center formation suggests that the replication cycle of the VZV was not completed. The VZV-infected B cells incorporated a mean of 2×10^3 counts of ^{51}Cr per 2,500 cells, and their spontaneous ^{51}Cr release was <15% after over 4 h of culture

TABLE 1. Lysis of VZV-infected B-cell lines by autologous T cells

Effector	% Specific lysis at effector/target ratios of[a]:			
	40:1	20:1	10:1	5:1
Fresh MNC	5 ± 2	5 ± 2	1 ± 1	3 ± 1
Stimulated MNC				
1 week	7 ± 2	5 ± 2	1 ± 1	3 ± 1
2 weeks	8 ± 3	5 ± 3	3 ± 1	3 ± 1
3 weeks	21 ± 4	18 ± 4	11 ± 3	1 ± 1
4 weeks	22 ± 3	18 ± 3	14 ± 4	8 ± 2

[a] Results are expressed as the mean plus or minus one standard error. Cells were stimulated with VZV and expanded with weekly restimulation and dilution in medium containing IL-2 before testing.

TABLE 2. Characteristics of VZV-specific T-cell clones after first cloning.[a]

Clone	Thymidine uptake		% Specific lysis	
	Control cells	Stimulated cells	Uninfected cells	VZV-infected cells
1	170	3,121	2	25
2	186	5,220	1	20
3	210	4,790	1	29
4	115	3,991	3	53
5	205	1,219	0	52
6	180	1,920	9	6
7	184	2,414	2	24
8	147	5,740	1	27
9	511	4,407	0	17

[a] Nine clones were obtained by limiting dilution of Percoll gradient-enriched, VZV-stimulated T-cell blast cells. Thymidine uptake was by 10^4 cells with 10^5 irradiated MNC as antigen-presenting cells with (stimulated cells) or without (control cells) VZV antigen. Specific lysis of uninfected (control) or VZV-infected autologous B-lymphoblastoid cells was in a 4-h ^{51}Cr release assay at a 40:1 effector ratio.

and 44% after 18 h. The EBV-transformed B cells could also be superinfected with CMV, as judged by the appearance of CMV cell surface antigens by immunofluorescence 3 days after the addition of live CMV. The B-cell lines were permissive for HSV, as shown by a >10-fold increase in virus titer in an infectious center assay over the 24 h after HSV infection.

Lysis of VZV-infected B-cell lines by T-cell blasts. VZV-infected and uninfected B-cell line cells were cultured with autologous MNC which were either freshly isolated or prestimulated with VZV. MNC that had been freshly isolated or stimulated with VZV 1 to 2 weeks previously did not lyse the targets (Table 1), but after 3 weeks of culture, low levels of lysis were consistently detected. These results suggest that VZV-infected B lymphoblasts were substantially less susceptible to lysis by MNC than were VZV-infected fibroblasts. The failure to achieve above 25% specific lysis might have been due to a low frequency of cytotoxic precursors in the cell line or it might have resulted from an intrinsic resistance to lysis of the target cells. To distinguish between these possibilities, we cloned the T cells as described in the Methods section. Nine clones were isolated, in each case from wells which had been seeded at 10 cells per well. There was no growth from wells seeded at 1 cell per well. All of the clones were $T3^+$ $T4^+$ $T8^-$ Tac^+ by immunofluorescence: their proliferative and cytotoxic characteristics are summarized in Table 2. Because these clones had all been isolated from wells which had been seeded at 10 cells per well, they were recloned at 1 cell per well before further specificity studies were undertaken.

Specificity of target cell lysis. Five clones (numbers 2, 4, 5, 8, and 9 in Table 2) were recovered from the recloning. Their specificity for virus was analyzed by testing the clones on targets infected with different herpesviruses. Clones 2, 4, 5, and 8 lysed only VZV-infected targets. Clone 9 lysed HSV-infected targets as well as VZV-infected targets (Fig. 2, cf. clone 9 with clones 4 and 8).

The specificity of the clones for MHC was tested with EBV lines and fibroblasts prepared from different donors and by cold target inhibition. Clones 4, 8, and 9 lysed autologous (HLA DR 3,4) VZV-infected targets but not VZV-infected fibroblasts matched with the T cells for HLA A2, A3, B7, and B12 (Fig. 3). Clones 2 and 5 also failed to lyse the same VZV-infected HLA-matched or -mismatched fibroblasts, and none of the clones lysed VZV-infected B-cell line cells of HLA DR 2,7, 4,5, or 4,7 types. The only unrelated B-cell line which was lysed by the clones was DR 3,4 positive (Table 3).

The addition of any cold targets to the cultures resulted in some inhibition of lysis, as was expected. Comparison between the cold targets used shows that the VZV-infected autologous B-cell line reduced the lysis of the labeled targets more than either of the other cold targets (Fig. 4 shows results with clone 8; similar results were obtained with clones 4 and 5). The preferential lysis of autologous rather than unrelated B-cell line targets in these experiments might have resulted from MHC restriction or from a special susceptibility of the target cells to autologous T cells. We therefore compared the ability of monoclonal antibodies to HLA class I and class II determinants to inhibit target cell lysis. The results (Fig. 5) indicate a greater inhibition of lysis

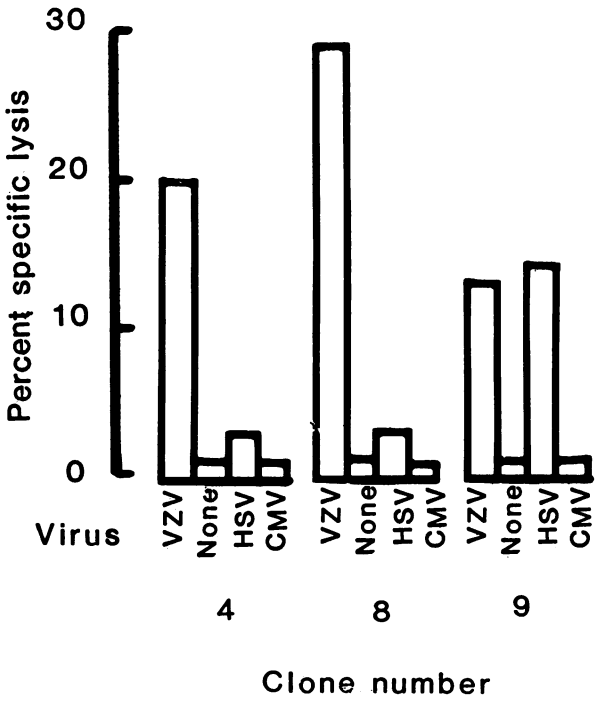

FIG. 2. Specificity of target cell lysis. The EBV-transformed B-cell targets are autologous and the percent specific lysis is at a 40:1 effector cell/target cell ratio.

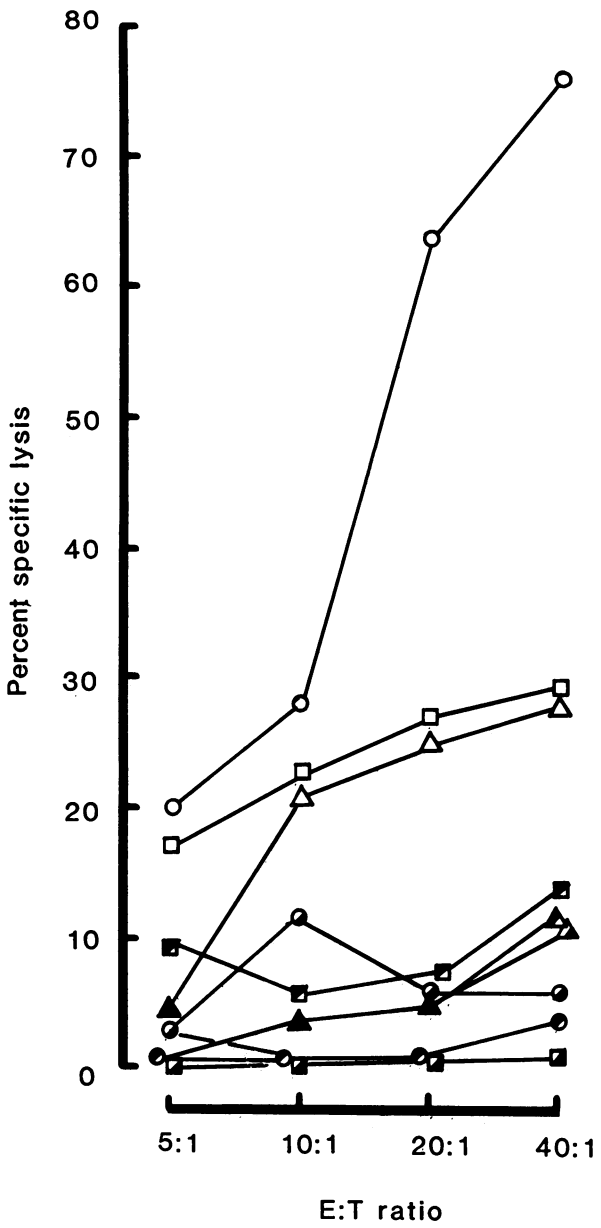

FIG. 3. Specificity of target cell lysis. Targets (with clones 4, 8, and 9, respectively, as the effector cells) were autologous VZV-infected B-cell line cells (□, ○, △), partially matched VZV-infected fibroblasts (◩, (◐, ▲), and autologous uninfected B-cell line cells (◪, ◑, ▲). E:T ratio, Effector/target ratio.

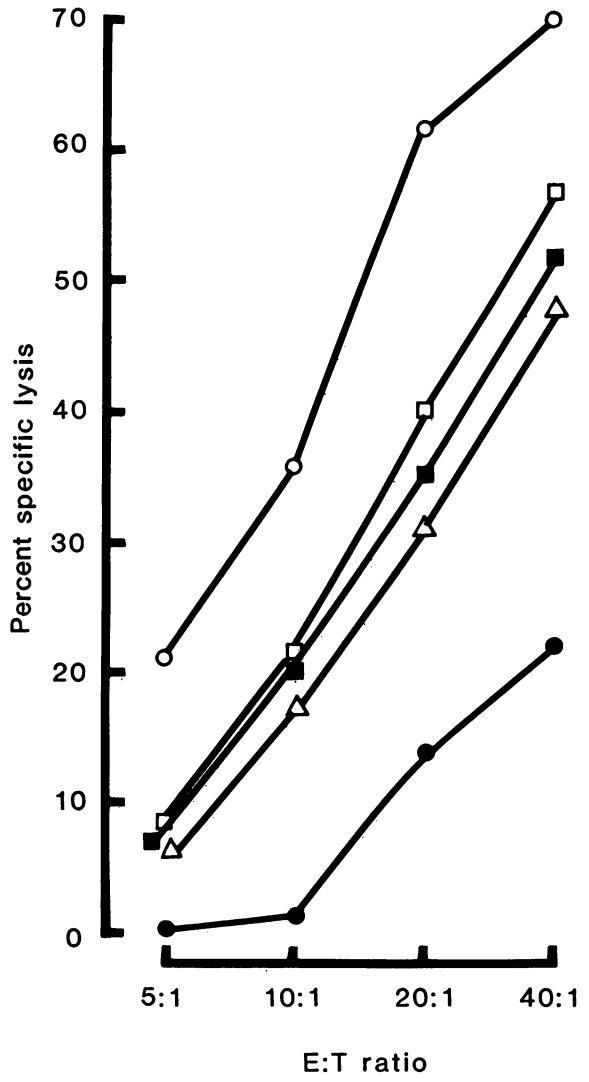

FIG. 4. Cold target inhibition of specific lysis. Clone 8 effector T cells with autologous VZV-infected, ^{51}Cr-labeled targets (DR 3,4) and no competing targets (○), unrelated (DR 4,5) uninfected competitors (■), unrelated (DR 4,5) VZV-infected competitors (□), autologous VZV-infected competitors (●), and autologous uninfected competitors (△). Ratio of ^{51}Cr-labeled targets to cold competitors, 1:10. E:T ratio, Effector/target ratio.

by the anti-HLA-DR compared with that obtained with the anti-class I antibody with clone 4 effectors. Similar results were obtained with clone 5 and 8 effectors. No inhibition of lysis was obtained with the anti-HLA-DQ antibody, Leu 10.

DISCUSSION

In our previous studies of cell-mediated lysis of VZV-infected fibroblasts, we invariably found nonspecific lysis when prestimulated T cells were the effectors (2). Target cell lysis in our present study is, in contrast, specific both for virus and an HLA DR (class II) histocompatibility antigen. Although the DR restriction appeared to be exclusively to DR 3, these data should be approached with caution because they rely on serological determination of the target cell antigens. Restriction fragment mapping indicates that there is heterogeneity within the serologically defined groups, as, for example, with DR 4 (7). Heterogeneity of this type could account for our failure to find any DR 4-restricted T-cell clones. Of five clones studied in detail, one lysed target cells infected with either VZV or HSV, and none lysed CMV-infected targets. The cross-reactivity with HSV is not totally unexpected, as HSV gB shares antigen determinants with VZV gp63 (6). Only clone 6 of the nine clones originally isolated showed any lysis of uninfected targets, and this was less than 10%. The specificity of this clone was not further tested in this study, but it is conceivable that it was responding to an EBV-derived antigen such as lymphocyte-derived membrane antigen (LYDMA) (10). The predominant specificity of the clones for VZV-associated antigens is not surprising in that they were originally isolated from a culture stimulated with glutaraldehyde-fixed, VZV-infected fibro-

TABLE 3. Lysis of autologous and unrelated VZV-infected B-cell lines

Target	Lysis with[a]:		
	No added antibody	Anti-DR	Anti-DQ
DR 3,4	62 ± 7	12 ± 3	66 ± 8
DR 2,7	8 ± 5	6 ± 5	8 ± 5
DR 4,5	4 ± 5	8 ± 3	6 ± 2
DR 4,7	10 ± 6	7 ± 6	8 ± 3
Autologous	74 ± 7	12 ± 4	77 ± 5

[a] Results are expressed as percent specific lysis at a 40:1 effector/target ratio and are calculated as the mean plus or minus one standard error for four clones. The final dilution of anti-DR antibody was 1:2,000; that of anti-DQ was 1:50. The autologous targets were autologous HLA DR 3,4 B lymphoblasts. Lysis of uninfected targets was <10% in all combinations.

blasts. In keeping with our previous observation (O. Pontesilli, M. Laszlo, M. Levin, and A. R. Hayward, Fed. Proc. 44:4514, 1985), most of the T cells responding to this stimulus were $T4^+$ and required antigen-presenting cells for their continued proliferation. The clones, before and after subcloning, proliferated in response to VZV antigen presented by autologous, irradiated blood MNC. Their ability to lyse VZV-infected targets is consistent with previous reports of target cell lysis by $T4^+$ cells (9, 12). Whether sufficient DR-positive cells become infected in vivo during the course of a VZV infection for target cell lysis by $T4^+$ cells to be an important defense mechanism is not known. There is, however, no reason to suppose that the T-cell clones we tested would be functionally restricted to cytotoxicity or unable to help B lymphocytes. MNC, either freshly isolated or after 1 to 2 weeks culture with VZV antigens, were not able to lyse the VZV-infected B-cell line cells in the 4-h isotope release assay we used in this series of experiments. It therefore appears that the B-lymphoblastoid lines are relatively resistant to lysis by natural killer cells, even when they have been activated in a VZV-stimulated culture. This contrasts with the marked susceptibility of VZV-infected fibroblasts to lysis by natural killer cells. The relatively low frequency of $T4^+$ VZV-specific T cells in blood (around 1:12,000 [3]) may account for the lack of lysis of the VZV-infected B cells by the fresh and 7-day-stimulated MNC.

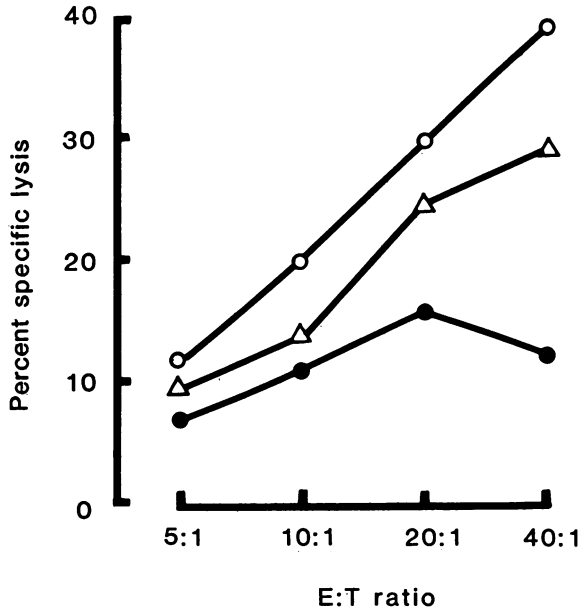

FIG. 5. Inhibition of lysis of autologous VZV-infected B-cell line cells by anti-DR antibody. Symbols: ○, control; △, with anti-HLA A/B framework antibody; ●, anti-DR antibody. Clone 4 cells were used as effectors. E:T ratio, Effector/target ratio.

EBV-transformed B-cell lines were infected with HSV and used as targets for cloned $T4^+$ T cells by Yasukawa and Zarling (17). Our own results indicate that the B-cell line cells are permissive for HSV, while they do not appear to support the replication of VZV. Evidence that VZV antigens were indeed expressed on the B-cell surface after infection with VZV comes from the cell surface immunofluorescence studies. (Fig. 1) and the specificity of the cytotoxicity we observed. The extent to which the VZV genome is expressed in the B-cell line cells is currently under investigation, but it would appear to be incomplete in that the VZV-superinfected cells had a low level of spontaneous lysis and they carried little virus in an infectious center assay. The in vitro survival of VZV-superinfected B cell lines should facilitate the preparation of VZV-specific T-cell blasts, because EBV-induced B-cell lines themselves function as antigen-presenting cells (4) obviating a need for monocytes.

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