Epstein-Barr Virus (EBV) Antigens Processed and Presented by B Cells, B Blasts, and Macrophages Trigger T-Cell-Mediated Inhibition of EBV-Induced B-Cell Transformation

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Received 6 September 1989/Accepted 20 November 1989

The ability of B cells, B blasts, and macrophages to present Epstein-Barr virion antigens to autologous T cells and trigger their capacity to inhibit Epstein-Barr virus-induced B-cell transformation was tested. Macrophages were as efficient as B cells and B blasts in presenting the virus to T lymphocytes. This function required antigen processing, because it was inhibited by chloroquine treatment and by fixation of the antigen-presenting cells immediately after viral exposure but not 18 h later. T cells exposed to the purified Epstein-Barr virus envelope antigen gp350 coupled to immunostimulating complexes also showed inhibitory function. These results suggest that recognition of processed virion antigens elicits the generation of T-cell-mediated inhibition of Epstein-Barr virus-induced B-cell transformation.

The demonstration that T lymphocytes from Epstein-Barr virus (EBV)-seropositive individuals inhibit the EBV-induced transformation of B cells in vitro has strongly influenced the current view on the role of cellular immunity in the control of EBV infection (23). The antigen(s) that triggers T cells for growth-inhibitory capacity in this in vitro system has not been identified. The findings that T cells inhibit the proliferation of autologous EBV-transformed lymphoblastoid cell lines, which do not enter the productive cycle (22), and that T cells isolated from regressing cultures lyse the lymphoblastoid cells have focused the attention to the restricted set of viral gene products that are regularly expressed in the EBV-transformed lymphoblastoid cells. These include six nuclear antigens (EBNA 1 through 6) and two membrane proteins, the latent membrane protein, and the membrane protein encoded by spliced exons from the terminal repeats (19, 25, 31). The contribution of cellular immunity against EBV structural antigens was studied only recently (29). We have shown that virion antigens presented by B lymphocytes can trigger the capacity of T cells to inhibit B-cell transformation (2). We have now examined the capacity and requirements of B cells, B blasts, and macrophages to present EBV virion antigens to autologous T lymphocytes.

Lymphocytes obtained from buffy coats of EBV-seropositive donors were separated into T- and B-cell-enriched populations by nylon wool passage and sheep erythrocyte rosetting as previously described (13). Samples of B cells were either frozen for later infection or exposed to blastogenic concentrations (1/40,000 dilution from a packed pellet) of Formalin-fixed *Staphylococcus aureus*. To obtain monocyte-macrophage-enriched populations, mononuclear cells were layered on top of Nycodenz-Monocytes (Nyegaard & Co., Oslo, Norway). The gradients were centrifuged for 15 min at 600 × g, and monocytes were recovered from the interface region. This population contained >95% macrophages as determined by latex ingestion or by morphology

evaluation on Giemsa-stained preparations. Macrophages, B cells, and B blasts were exposed for 1 h at 37°C to UV-inactivated EBV preparations (2). T cells were cultured in RPMI 1640 (Flow Laboratories, Inc.; no. 12602) supplemented with heat-inactivated fetal calf serum, 100 µg of streptomycin per ml, 100 U of penicillin per ml, and 2 mM L-glutamine either alone or with 4,000-rad-irradiated antigen-presenting cells (APC) at a T/B or T/macrophage cell ratio of 10:1. Three days later, T cells were recovered from the various cultures and assayed for their ability to inhibit the growth of the freshly infected autologous B cells as previously described (13). The strength of the outgrowth inhibition is expressed as the regression index, representing the minimum number of T cells required for a 50% reduction of B-lymphocyte growth.

T lymphocytes recovered from the mixed cultures containing different types of APC that had been pre-exposed to UV-inactivated virus inhibited with similar efficiencies the transformation of freshly EBV-infected B cells. The inhibitory effect was evident from the comparison with the effect of T cells cultured alone or with APC that had not been exposed to the virus. It is noteworthy that T cells exposed to uninfected B blasts did not generate inhibitory activity. It seems, therefore, that T-cell responses acting in the transformation system are directed to viral antigens presented by the infected cell and not to B-cell- or B-blast-specific antigens as previously suggested (14).

Macrophages and B blasts were as efficient as B lymphocytes in presenting the virus to the T cells (Fig. 1). Thus, a role of macrophages in the EBV-specific cellular immunity is shown here for the first time. We verified the absorption of virus to macrophages by measuring the residual infectivity of viral preparations that had been preincubated with various numbers of macrophages. This procedure resulted in a dose-dependent reduction of EBNA-inducing potential (Table 1). Although the EBV receptor CR2/CD21 was initially thought to be present only on B lymphocytes, it is now established that it is present in other cell types (3, 28-34), including activated macrophages (6, 9). Whether the inter-

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FIG. 1. Presentation of inactive virus by B cells, B blasts, and macrophages: growth-inhibitory capacity of T cells cultured for 3 days alone (T), with autologous noninfected B cells (T+B), B blasts (T+B blast), or macrophages (T+M ϕ), or with UV-inactivated B95-8 virus-infected B cells (T+B_v), B blasts (T+B blast_v), or macrophages (T+M ϕ _v). Mixed cultures containing 4×10^4 freshly EBV-infected B cells and graded numbers of T cells recovered from the 3-day-old cultures were set up in flat-bottomed microdilution plates. Four weeks later EBV-induced B-cell growth was evaluated. The results are expressed as the minimum number of T cells required for 50% inhibition of the EBV-induced B-lymphocyte proliferation. Shown are the means $\pm$ standard deviations of five experiments performed with different individuals.

action of EBV with the macrophages involves the specific CR2/CD21 receptor remains to be determined.

In other viral systems it has been demonstrated that antigens are processed and antigenic peptides are presented to the T cells in association with major histocompatibility complex (MHC) molecules (32). Processing requirements for antigen presentation vary between viruses and between major histocompatibility complex class I- and class II-restricted responses to the same virus (18). Most antigens must be at least unfolded, if not fragmented to reveal peptides that bind to major histocompatibility complex molecules. In our experiments the generation of inhibitory T cells could be due to recognition of virion-derived antigenic moieties processed by the presenting cells or to recognition of virus particles attached to the cell membrane after infec-

FIG. 2. Effect of chloroquine and glutaraldehyde fixation on the presentation of virus by B cells and macrophages: growth-inhibitory capacity of T cells cultured with autologous B cells or (T+B₉₅₈ UV) macrophages (T+M ϕ ₉₅₈ UV) infected with UV-inactivated B95-8 virus in the presence of chloroquine. The infected cells were fixed with glutaraldehyde either immediately (■) or 18 h after viral exposure (▨) or were not fixed (□). Shown are the means $\pm$ standard errors of three different experiments. The test for the T-cell effect on the autologous EBV-infected B cells is described in the legend to Fig. 1.

tion. To distinguish between these two possibilities, we examined the generation of outgrowth-inhibitory capacity under conditions in which antigen processing does not take place. Chloroquine, a lysosomal agent known to inhibit antigen processing, was added during the infection period. We chose a concentration of 50 μ M, which was shown to inhibit presentation of influenza virion antigens (18). This concentration did not affect the binding and penetration of EBV because it did not reduce the induction of EBNA in B cells and in the EBV-negative BJAB cell line (data not shown).

T lymphocytes were cultured with APC exposed to UV-inactivated virus in the presence of chloroquine and fixed immediately or 18 h later with glutaraldehyde. Fixation is known to inhibit antigen processing without influencing the capacity to present already processed antigens or antigenic peptides (8, 27). Accordingly, immediate fixation of APC abrogated the sensitization step. T cells derived from these mixed cultures had levels of inhibition similar to those of T cells cocultivated with noninfected APC. On the other hand, fixation of the APC 18 h after virus exposure did not impair their priming capacity; T cells cultured with such APC were as efficient in inhibiting B-cell growth as were T cells cocultivated with infected nonfixed APC (Fig. 2).

Among the structural components of the virion, the major envelope glycoprotein gp340 is likely to be responsible for T-cell sensitization. This viral glycoprotein interacts with the viral receptor on the B cells (20, 36), it is the target of neutralizing antibodies (7, 30), and it activates the alternative complement pathway (15). Antibodies to gp340 mediate antibody-dependent cellular cytotoxicity (21), and they inhibit virus release from productive cell lines (24). Cotton-top tamarins immunized with purified gp340 incorporated into immunostimulating complexes (iscoms) were protected

TABLE 1. Adsorption of virus to macrophages

Virus	% of EBNA-positive cells ^a		
	Expt 1	Expt 2	Expt 3
Control	21	25	19
Adsorbed to 3×10^6 macrophages ^b	4	10	9
Adsorbed to 1×10^6 macrophages ^b	11	17	ND ^c

^a Determined 72 h after infection of BJAB cells.

^b B95-8 virus (1 ml) was incubated for 90 min at 37°C with different numbers of purified macrophages, and the supernatants were collected and tested for induction of EBNA in BJAB cells.

^c ND, Not determined.

FIG. 3. Stimulation of T cells with gp340 iscoms: growth-inhibitory capacity of T cells cultured alone (T control) or stimulated with either gp340-iscoms (T+gp340 iscoms) or with iscoms matrix (T+matrix). As a control the outgrowth-inhibiting capacity of freshly separated T cells (T fresh) is shown. Shown are the means $\pm$ standard deviations of five different experiments. The test for the T-cell effect on the autologous EBV-infected B cells is described in the legend to Fig. 1.

against EBV-induced lymphoma. Interestingly, the levels of protection did not always correlate with levels of neutralizing antibodies (17). In a recent study gp340 iscoms were shown to be the target of EBV-specific major histocompatibility complex class II-restricted proliferating clones generated by stimulation of lymphocytes from EBV-seropositive individuals with UV-inactivated virus (33). These results prompted us to examine the capacity of gp340 to trigger T-cell-mediated inhibition of EBV-induced B-cell transformation.

gp340 was purified to homogeneity (as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) from B95-8 cell membranes by fast-protein liquid chromatographic ion exchange (5) and incorporated into iscoms (12, 17). These preparations were examined by electron microscopy and exhibited characteristic 35-nm-diameter cage-like structures (16). Specific binding of gp340 iscoms to EBV-receptor CR2-positive Raji cells was confirmed by indirect immunofluorescence. A preparation of quail A and cholesterol without gp340, exhibiting the typical cage-like structure was used as control (referred to as matrix in the figures) (12). Peripheral blood lymphocytes were exposed to gp340 iscoms or to matrix for 7 days. T lymphocytes from matrix-stimulated cultures exerted a weak inhibition similar to that of T cells derived from control cultures, i.e., peripheral blood lymphocytes cultured alone (regression indexes, 6.2 ± 1.2 and 7.1 ± 0.1 , respectively). T cells cultured with gp340 iscoms showed higher inhibitory capacity (regression index, 1.6 ± 0.7) than did T cells cultured with matrix ($P < 0.05$). The inhibitory effect of this population was similar to that of freshly separated T lymphocytes tested in parallel (regression index, 2.8 ± 0.7 ; $P > 0.1$) (Fig. 3).

Identification of virion antigens and antigens associated with lytic infection as targets for T-cell responses is of importance in the light of the current view on viral persistence. The lymphoid compartment represents the primary site for the maintenance of the virus in the infected hosts (10a). The facts that the spontaneous outgrowth of EBV-carrying lymphoblastoid cells from blood of EBV-seroposi-

tive individuals is largely dependent on the release of virus and subsequent in vitro infection of B cells (11) and that productive infection of epithelial cells is frequent in healthy virus carriers (37) support the idea that lytic infection and immune responses to antigens associated with the productive cycle of the virus may be more important than previously recognized in controlling and avoiding the manifestation of the transforming potential of B cells.

Our results show that T lymphocytes with specificity to virion antigens can counteract B-cell transformation. In vivo such T lymphocytes may control the lytic infection in the epithelium, as indicated by the fact that in conditions of immunosuppression the shedding of infectious virus in throat washings is elevated (38). Recent evidence showing that most epithelia contain resident lymphocytes expressing the $\gamma\delta$ T-cell receptor (1, 4) has led to the suggestion that $\gamma\delta$ -bearing T cells mediate the immunological surveillance function of epithelia (10). In addition, the virus-specific T lymphocytes may reduce the pool of infected B cells. Recent findings suggest that T cells may also prevent transformation by intracellular inactivation of the virus (26); this idea is supported by the demonstration that CD8-positive T cells inhibit human immunodeficiency virus replication by an apparently nonlytic mechanism that does not involve gamma interferon production (35).

This investigation was supported by Public Health Service grant 5RO1 CA 30264 awarded by the National Cancer Institute and by the Swedish Cancer Society.

Karin Kvarnung, Gill Ekstrom, and Barbro Ehlin-Henriksson provided excellent technical help.

LITERATURE CITED

- Asarnow, D. M., W. A. Kuziel, M. Bonyhadi, R. E. Tigelaar, P. W. Tucker, and J. P. Allison. 1988. Limited diversity of Yd antigen receptor genes of Thy-1⁺ dendritic epidermal cells. *Cell* 55:837-847.
- Bejarano, M. T., M. G. Masucci, G. Klein, and E. Klein. 1988. T-cell mediated inhibition of EBV-induced B-cell transformation: recognition of virus particles. *Int. J. Cancer* 12:359-364.
- Bhan, A. K., L. M. Nadler, P. Stashenko, R. T. McCluskey, and S. F. Schlossman. 1981. Stages of B cell differentiation in human lymphoid tissue. *J. Exp. Med.* 154:737-749.
- Bonneville, M., C. A. Janeway, Jr., K. Ito, W. Hasen, I. Ishida, N. Nakanishi, and S. Tonegawa. 1988. Intestinal intraepithelial lymphocytes are a distinct set of Yd T cells. *Nature (London)* 336:479-481.
- David, E. M., and A. J. Morgan. 1988. Efficient purification of Epstein-Barr virus membrane antigen gp340 by fast protein liquid chromatography. *J. Immunol. Methods* 108:231-236.
- Fearon, D. T. 1984. Cellular receptors for fragments of the third component of complement. *Immunol. Today* 5:105-110.
- Hoffman, G. J., S. G. Lazarowitz, and S. D. Hayward. 1980. Monoclonal antibody against a 250,000-dalton glycoprotein of Epstein-Barr virus identifies a membrane antigen and a neutralizing antigen. *Proc. Natl. Acad. Sci. USA* 77:2979-2983.
- Hosken, N. A., M. J. Bevan, and F. R. Carbone. 1989. Class I-restricted presentation occurs without internalization or processing of exogenous antigenic peptides. *J. Immunol.* 142:1079-1083.
- Inada, S., E. J. Brown, T. A. Gaither, C. H. Hammer, T. Takahashi, and M. M. Frank. 1983. C3d receptors are expressed on human monocytes after in vitro cultivation. *Proc. Natl. Acad. Sci. USA* 80:2351-2355.
- Janeway, C. M., B. Jones, Jr., and A. Hayday. 1988. Specificity and function of T cells bearing Yd receptors. *Immunol. Today* 9:73-76.
- 10a. Klein, G. 1989. Viral latency and transformation: the strategy of Epstein-Barr virus. *Cell* 58:5-8.
- Lewin, N., P. Aman, M. G. Masucci, E. Klein, G. Klein, B.

- Oberg, H. Strander, W. Henle, and G. Henle. 1987. Characterization of EBV-carrying B-cell populations in healthy seropositive individuals with regard to density, release of transforming virus and spontaneous outgrowth. *Int. J. Cancer* **39**:472-476.
12. Lovgren, K., and B. Morein. 1988. The requirement of lipids for the formation of immunostimulating complexes (iscoms). *Bio-technol. Appl. Biochem.* **10**:161-172.
13. Masucci, M. G., M. T. Bejarano, G. Masucci, and E. Klein. 1983. Large granular lymphocytes inhibit the in vitro growth of autologous Epstein-Barr virus-infected B cells. *Cell. Immunol.* **76**:311-321.
14. Masucci, M. G., and E. Klein. 1983. Role of T cell subpopulations in the control of the proliferative potential of EBV-transformed B cells. *Behring Inst. Mitt.* **72**:163-168.
15. Mold, C., B. M. Bradt, G. R. Nemerow, and N. R. Cooper. 1988. Activation of the alternative complement pathway by EBV and the viral envelope glycoprotein, gp350. *J. Immunol.* **140**:3867-3874.
16. Morein, B., B. Sundquist, S. Hoglund, K. Dalsgaard, and A. Osterhaus. 1984. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. *Nature (London)* **308**:457-460.
17. Morgan, A. J., S. Finerty, K. Lovgren, F. T. Scullion, and B. Morein. 1988. Prevention of Epstein-Barr (EB) virus-induced lymphoma in cottontop tamarins by vaccination with the EB virus envelope glycoprotein gp340 incorporated into immunostimulating complexes. *J. Gen. Virol.* **69**:2093-2096.
18. Morrison, L. A., A. E. Lukacher, V. L. Braciale, D. P. Fan, and T. J. Braciale. 1986. Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *J. Exp. Med.* **163**:903-921.
19. Moss, D. J., I. S. Misko, S. R. Burroughs, K. Burman, R. McCarthy, and T. B. Sculley. 1988. Cytotoxic T cell clones discriminate between A and B type Epstein-Barr virus transformants. *Nature (London)* **331**:719-721.
20. Nemerow, G. R., C. Mold, V. Keivens Schwend, V. Tollefson, and N. R. Cooper. 1987. Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and the C3 complement fragment C3d. *J. Virol.* **61**:1416-1420.
21. Qualtiere, L. F., R. Chase, and G. Pearson. 1982. Purification and biological characterization of a major Epstein-Barr virus-induced membrane glycoprotein. *J. Immunol.* **129**:814-818.
22. Rickinson, A. B., D. J. Moss, D. J. Allen, L. E. Wallace, M. Rowe, and M. A. Epstein. 1981. Reactivation of Epstein-Barr virus-specific cytotoxic T cells by in vitro stimulation with the autologous lymphoblastoid cell line. *Int. J. Cancer* **27**:593-601.
23. Rickinson, A. B., D. J. Moss, L. E. Wallace, M. Rowe, I. S. Misko, M. A. Epstein, and J. H. Pope. 1981. Long-term T-cell mediated immunity to Epstein-Barr virus. *Cancer Res.* **41**:4216-4221.
24. Sairenji, T., G. Bertoni, M. M. Medveczky, P. G. Medveczky, Q. V. Nguyen, and R. E. Humpreys. 1988. Inhibition of Epstein-Barr virus (EBV) release from P3HR-1 and B95-8 cell lines by monoclonal antibodies to EBV membrane antigen gp350/220. *J. Virol.* **62**:2614-2621.
25. Sample, J., D. Liebowitz, and E. Kieff. 1989. Two related Epstein-Barr virus membrane proteins are encoded by separate genes. *J. Virol.* **63**:933-937.
26. Sellins, K. S., and J. J. Cohen. 1989. Polyomavirus DNA is damaged in target cells during cytotoxic T-lymphocyte-mediated killing. *J. Virol.* **63**:572-578.
27. Shimonkevitz, R., J. Kappler, P. Marrack, and H. Grey. 1983. Antigen recognition by H-2 restricted T cells: cell free antigen processing. *J. Exp. Med.* **158**:303-316.
28. Sixbey, J. W., E. H. Vesterinen, J. G. Nedrud, N. Raab-Taub, L. A. Walton, and J. S. Pagano. 1983. Replication of Epstein-Barr virus in human epithelial cells infected in vitro. *Nature (London)* **306**:480-483.
29. Sugamura, K., Y. Tanaka, and Y. Hinuma. 1982. Expression of target antigen for Epstein-Barr virus-specific cytotoxic T cells on BJAB cells freshly infected with EBV. *Microbiol. Immunol.* **26**:575-583.
30. Thorley-Lawson, D. A., and K. Geling. 1980. Monoclonal antibodies against the major glycoprotein (gp350/320) of Epstein-Barr virus neutralize infectivity. *Proc. Natl. Acad. Sci. USA* **77**:5307-5311.
31. Thorley-Lawson, D. A., and E. S. Israelsohn. 1987. Generation of specific cytotoxic T-cells with a fragment of the Epstein-Barr virus-encoded P63/latent membrane protein. *Proc. Natl. Acad. Sci. USA* **88**:5384-5388.
32. Townsend, A., F. Gotch, and J. Davey. 1985. Cytotoxic T cell recognize fragments of the influenza nucleoprotein. *Cell* **42**:457-467.
33. Ulaeto, D., L. Wallace, A. Morgan, B. Morein, and A. B. Rickinson. 1988. In vitro T cell responses to a candidate Epstein-Barr virus-vaccine: human CD4⁺ T cell clones specific for the major envelope glycoprotein gp340. *Eur. J. Immunol.* **18**:1689-1697.
34. Vik, D. P., and D. T. Fearon. 1985. Neutrophils express a receptor for iC3b, C3dg and C3d that is distinct from CR1, CR2 and CR3. *J. Immunol.* **134**:2571-2579.
35. Walker, C. M., D. J. Moody, D. P. Stites, and J. A. Levy. 1986. CD8⁺ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* **234**:1563-1566.
36. Wells, A., N. Koide, and G. Klein. 1982. Two large virion envelope glycoproteins mediate Epstein-Barr virus binding to receptor-positive cells. *J. Virol.* **41**:286-297.
37. Yao, Q. Y., A. B. Rickinson, and M. A. Epstein. 1985. A re-examination of the Epstein-Barr virus carrier state in healthy seropositive individuals. *Int. J. Cancer* **35**:35-42.
38. Yao, Q. Y., A. B. Rickinson, and M. A. Epstein. 1985. In vitro analysis of the Epstein-Barr virus:host balance in long term renal allograft recipients. *Int. J. Cancer* **35**:43-49.