Leukocyte Migration Inhibition Demonstrates a Human T-Cell Response to a Membrane Protein Expressed in Latent Epstein-Barr Virus Infection

DOV SULITZEANU,^{1*} ROBERT SZIGETI,² GEORGE KLEIN,² KEVIN HENNESSY,³ AND ELLIOTT KIEFF³

Lautenberg Center for General and Tumor Immunology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel¹; Department of Tumor Biology, Karolinska Institute, S-104 01 Stockholm, Sweden²; and Kovler Viral Oncology Laboratories, The University of Chicago, Chicago, Illinois 60637³

Received 5 June 1985/Accepted 6 December 1985

Leukocyte migration inhibition tests show that lymphocytes of Epstein-Barr virus-seropositive individuals recognize a Raji cell membrane antigen and a membrane protein encoded by Epstein-Barr virus in latently infected cells. Antiserum against the latter blocks the leukocyte migration inhibition triggered by both preparations, suggesting that the two antigens are associated with the same protein complex.

After primary human infection, Epstein-Barr virus (EBV) persists in latently infected B lymphocytes that have increased proliferative capacity. It is widely believed that the proliferation of EBV-transformed B cells *in vivo* is under the control of EBV-specific cytotoxic T cells (3) which recognize an antigen operationally designated as LYDMA (lymphocyte-detected membrane antigen [4]). Since it is generally difficult to inhibit T-cell cytotoxicity with antibodies against the target antigen, no human or animal antisera against LYDMA have been produced and, consequently, the identity of LYDMA has not yet been established. A potential candidate was suggested recently, based on experiments in which T cells of EBV-seropositive individuals were found to recognize a membrane antigen of EBV-transformed B cells by the leukocyte migration inhibition (LMI) technique (7). However, the molecule responsible for triggering the LMI reaction was not identified.

Three viral mRNAs, two of which encode EBV-specific nuclear antigens (1) have been recently identified in latently infected, growth-transformed cells. The third and most abundant of these mRNAs encodes a protein with the amino half consisting predominantly of six highly hydrophobic domains, which are likely to insert the protein into the cell membrane. The carboxy half of the protein is hydrophilic and highly negatively charged. This latter domain has been expressed in *Escherichia coli* as a fusion protein with the amino terminus of β -galactosidase. A rabbit antiserum to this EBV-encoded, latently infected cell membrane protein identified a polypeptide in immunoblots of membranes of latently infected, growth-transformed cells (2). In immunofluorescence experiments, the antiserum reacted with the periphery of fixed but not live cells, confirming the initial hypothesis that the carboxy half of the protein is oriented towards the cell interior (2). Since EBV-specific nuclear antigen molecules are not associated with the cell membrane (2), the EBV-encoded membrane protein is a likely candidate as a target for cytotoxic T cells, i.e., LYDMA.

We now report the first evidence of a human cellular immune response to the EBV-encoded membrane protein, by the LMI assay. The rabbit antiserum to the latent

membrane protein blocks the LMI response to the protein and to latently infected Raji cell membranes, indicating the association of the EBV-specified protein with the antigenic reactivity of latently infected cell membranes.

Raji cells, derived from an EBV-positive Burkitt's lymphoma were used to prepare LMI-inducing antigen. Ramos cells, derived from an EBV-negative Burkitt's lymphoma, and EBV-infected Ramos cells (AW Ramos) (7) were used to absorb antisera to EBV-encoded membrane protein.

Raji cells suspended in 20 mM Tris buffer (pH 7.4) were disrupted in a Dounce homogenizer, and the homogenate was ultracentrifuged at $100,000 \times g$ for 1 h. The supernatant, designated R1, was removed, and the sediment was first extracted with 0.1% and then with 1% Nonidet P-40. The second extract, R3, was ultracentrifuged and dialyzed (for full details, see reference 7). The fusion protein and the corresponding rabbit antiserum were prepared as described previously (2). LMI assays were carried out by the direct agarose microdroplet technique (5). Briefly washed buffy coat cells (20×10^6) were mixed with 135 μ l of nutrient agarose medium containing an equal volume of $2 \times$ RPMI 1640 medium, supplemented with 20% horse serum and 0.4% agarose. Droplets (2 μ l) were placed in triplicate in migration chambers (Sterilin) and left to solidify, after which the chambers were filled with medium with or without the antigen and incubated in a 37°C humidified CO₂ atmosphere for 24 h. The migration areas were then measured, and the results were expressed as the mean migration index (MI), by the formula: $MI = M_t/M_c$, where M_t is the mean migration area with antigen and M_c is the mean migration area of control cultures. An inhibition of at least 20% is generally regarded as significant in such assays.

The ability of the rabbit antiserum to the fusion protein to block the LMI induced by R1 and R3 was tested as follows. The antiserum and a control, normal rabbit serum, diluted 1/10, were absorbed with an equal volume of a mixture of packed, glutaraldehyde-fixed Molt 4 and K562 cells, the absorbed antisera were mixed with suitable amounts of R1 or R3, and the mixtures were kept overnight at 4°C. The next day, the mixtures were either assayed in LMI tests directly or treated first with *Staphylococcus aureus* Cowan I for 30

* Corresponding author.

FIG. 1. LMI induced by the EBV-encoded, latently infected cell membrane protein. Symbols: ○, cells from EBV-seronegative donors; ●, cells from EBV-seropositive donors; ■ and □, blocking experiments with leukocytes of EBV-seropositive donors in the presence of rabbit antiserum to the EBV-encoded membrane protein and nonimmune rabbit serum at 1/1,000 dilution, respectively. The means plus standard error of seven experiments are shown. The difference in the response of seronegative and seropositive donors at 20 µg of protein per ml was statistically significant ($P < 0.01$, Student's test).

min on ice (4 µl of packed bacteria per 1 µl of the antiserum) to remove the immune complexes. In the latter case, the bacteria were sedimented by centrifugation, and the supernatants were diluted in medium and then filtered through membrane filters (pore size, 0.45 µm; Millipore Corp.). The percentage of blocking was calculated by the formula: $(MI_0 - MI_1)/MI_0 \times 100$, where MI_0 is the mean MI in the culture

medium and MI_1 is the mean MI in the presence of the test antiserum.

To examine the capacity of various cells to absorb out the blocking activity of the antiserum, the latter was first preabsorbed with Molt 4 and K562 cells (see above). Amounts (200 µl) were then added to 100 µl of packed, glutaraldehyde-fixed Ramos or AW Ramos cells. The tubes were rotated at 4°C for 3 h, the absorbed sera were mixed with suitable antigen dilutions, and the mixtures were left to stand overnight at 4°C before testing for LMI activity.

When incubated with buffy coat cells from EBV-immune (seropositive) and -nonimmune (seronegative) individuals in the direct LMI assay, the fusion protein induced a significant migration inhibition, and this reaction was effectively blocked by the rabbit antiserum (Fig. 1). About 100-fold-larger concentrations of the fusion protein as compared with the Raji cell membrane fraction were required to trigger a reaction of similar intensity (7). Similar concentrations of purified β-galactosidase did not induce LMI with either the immune or the nonimmune buffy coat leukocytes (data not shown).

Since T lymphocytes are believed to be the source of the leukocyte migration inhibition factor in antigen-induced LMI assays (6), these data suggest that there is a human T-cell immune response to the epitopes expressed on the EBV-encoded membrane protein. Preliminary experiments (unpublished data) showed that the nylon wool-nonadherent cells that form rosettes with sheep erythrocytes are essential for the migration inhibition seen when peripheral blood leukocytes of seropositive persons are exposed to either Raji cell membrane fractions or the EBV-encoded cell membrane protein, similar to what was found previously with the LMI induced by the EBV-determined nuclear antigen (6).

The rabbit antiserum to the fusion protein specifically abolished most of the LMI effect of the Raji cytosol and membranes (Fig. 2) whether or not the immune complexes

FIG. 2. Blocking of Raji cell fraction-induced LMI by the rabbit antiserum to the EBV-encoded, latently infected cell membrane protein. The means plus standard error of three experiments are presented. MIs are shown as columns. ■, The LMI without additional serum; □ and ▨, experiments in the presence of immune and normal control rabbit serum, respectively.

FIG. 3. Absorption of the blocking activity of the rabbit antiserum to the EBV-encoded, latently infected cell membrane protein with intact, glutaraldehyde-fixed cells. The Raji cell fractions were tested at final concentrations of 10 and 5 µg/ml. Final dilutions of the rabbit antiserum were 1/500 and 1/1,000. Two LMI experiments (horizontal line in the columns) are presented: with antigen alone (■) and with antigen in the presence of rabbit antiserum preabsorbed with Ramos cells (□) or with AW Ramos cells (▤).

had been removed by treatment with staphylococci. This indicates that the predominant immune response in the LMI assay is directed to the EBV protein. Furthermore, the blocking activity was specific, since EBV-specific nuclear antigen-induced LMI was not blocked by the antiserum (control experiments, data not shown). Blocking of the LMI could be reversed by preincubating the antiserum with glutaraldehyde-fixed, EBV-converted Ramos cells (AW Ramos) but not with EBV-negative Ramos cells (Fig. 3). The ability of the rabbit antiserum to block the LMI induced by Raji cell fractions R1 and R3 moieties are similar or identical. This conclusion is reinforced by the findings that only the converted AW Ramos (and not Ramos) cells could absorb the blocking activity of the antiserum.

Our results show that EBV-sensitized T cells recognize a virus-encoded protein associated with the membrane of EBV-transformed cells. Since this is the only virally coded membrane antigen believed to be expressed in latently infected cells, it would not be unreasonable to speculate that the antigen recognized in the LMI assay might also be the target of EBV-specific cytotoxic cells, i.e., LYDMA. It must be stressed, however, that the antigen identified by the LMI test cannot be equated directly with the target of the cytotoxic tests, since the two assays measure two distinct phenomena. Moreover, the fusion protein studied here includes only the intracellular domain of the EBV-encoded membrane protein (2). Consequently, it cannot be seen by the cytotoxic T cells on the surface of the EBV-transformed cells. Further work will be necessary to establish the relationship (if any) between the EBV-encoded membrane protein and LYDMA. Exposure of target cells, transfected with an EBV subgenomic fragment that encodes the membrane antigen, to human lymphocyte antigen-matched, EBV-sensitized T cells in cytotoxic experiments may be the most direct approach to this question.

This work was supported by the Fund for Basic Research, administered by the Israel Academy of Sciences and Humanities; by a grant from Concern II, Young Adults Funding Cancer Research, Los Angeles, Calif.; by Public Health Service grant 2R01CA 30264-04 from the National Institutes of Health; by the Swedish Cancer Society; and by grant MU 32H from the American Cancer Society and the Cancer Research Foundation of Chicago.

Robert Szigeti is a recipient of a fellowship from Cancer Research Institute, Inc., New York, New York, and the Concern Foundation, Los Angeles, Calif.

LITERATURE CITED

1. Fennewald, S., V. van Santen, and E. Kieff. 1984. Nucleotide sequence of an mRNA transcribed in latent growth-transforming virus infection indicates that it may encode a membrane protein. *J. Virol.* 51:411-419.
2. Hennessy, K., S. Fennewald, M. Hummel, T. Cole, and E. Kieff. 1984. A membrane protein encoded by Epstein-Barr virus in latent growth transforming infection. *Proc. Natl. Acad. Sci. USA* 81:7207-7211.
3. Rickinson, A. B., and D. J. Moss. 1983. Epstein-Barr virus induced transformation: immunological aspects. *Adv. Viral Oncol.* 3:213-238.
4. Svedmyr, E., and M. Jondal. 1975. Cytotoxic effector cells specific for B cell line transformed by Epstein-Barr virus are present in patients with infectious mononucleosis. *Proc. Natl. Acad. Sci. USA* 72:1622-1626.
5. Szigeti, R., J. Luka, and G. Klein. 1981. Leukocyte migration inhibition studies with Epstein-Barr virus (EBV)-determined nuclear antigen (EBNA) in relation to the EBV-carrier status of the donor. *Cell. Immunol.* 58:269-277.
6. Szigeti, R., G. Masucci, B. Ehlin-Henriksson, K. Bendtzen, G. Henle, W. Henle, G. Klein, and E. Klein. 1984. EBNA-specific LIF production of human lymphocyte subsets. *Cell. Immunol.* 83:136-141.
7. Szigeti, R., D. Sulitzeanu, G. Henle, W. Henle, and G. Klein. 1984. Detection of an EBV associated membrane antigen in EBV-transformed virus nonproducer cells by leukocyte migration inhibition and blocking antibody. *Proc. Natl. Acad. Sci. USA* 81:4178-4181.