Epstein-Barr Virus-specific Leukocyte Migration Inhibition Reaction with Tumor Biopsy Extracts: Correlation with the Presence of Viral DNA

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ABSTRACT

The Epstein-Barr virus (EBV)-specific leukocyte migration inhibition (LMI) reaction was used to detect EBV antigens in human tumor biopsies in parallel with nucleic acid hybridization for EBV DNA. None of six EBV DNA-negative tumors gave any significant LMI reaction. Fourteen of 17 EBV DNA-positive tumors gave a significant difference between the migration of leukocytes from EBV-seropositive versus -seronegative donors. One tumor gave a borderline reaction. The two LMI-negatives in this group had only a marginal EBV DNA content. It is suggested that the EBV-specific LMI test may be useful for detecting EBV genomes in tissue and tumor extracts.

INTRODUCTION

Recently, we have demonstrated that the leukocytes of EBV-seropositive (SP) healthy persons respond with a LMI reaction when confronted with extracts of EBV genome-carrying cell lines but not with corresponding EBV-negative cell extracts. The leukocytes of SN do not respond to any of the extracts (25, 29). We have also shown that purified EBNA can induce such a response by itself (26). It was also possible to demonstrate specific LMI reactions against the antigens associated with the cycle of the EBV:EA, and VCA. EA- and VCA-specific LMI reactions could occur in the absence of any EBNA-specific LMI response in acute infectious mononucleosis, in certain immunodeficiencies, and during immunosuppression (27).

In addition to the 2 well-known EBV-carrying human tumors, Burkitt's lymphoma and NPC, multiple EBV genomes were detected recently in a variety of lymphoproliferative lesions that occurred in patients with certain congenital or iatrogenic immunosuppression (27).

Nucleic acid hybridization can be highly reliable if performed under appropriate conditions, but it is not available to laboratories involved in immunological studies on the interaction between EBV, its target cell, the human B-lymphocyte, and the human host. The LMI reaction, an in vitro correlate of delayed-type hypersensitivity (3, 24), is, on the other hand, readily available for any immunological laboratory. Since EBV DNA-containing cells are also believed to contain EBV antigens, the nuclear antigen EBNA in particular, we have therefore asked the question, whether the LMI test, performed with leukocytes of healthy SP donors, can also detect EBV-associated antigens in tumor biopsies, not only cell lines. As a model system, we have chosen the 2 well-known EBV-carrying tumors, Burkitt's lymphoma and NPC, to prepare antigenic extracts. Non-EBV-associated tumors served as parallel controls. LMI tests were done in parallel with nucleic acid hybridizations to detect EBV DNA.

MATERIALS AND METHODS

Leukocyte Donors. Four EBV-SN and 20-SP healthy volunteers donated responder leukocytes. SN donors gave no detectable reaction at a minimum dilution of serum at 1:10 in the EA and VCA test and at 1:2 in the EBNA test. All SP donors had antibodies to VCA IgG (>40) and EBNA (>2), but not to EA (<10). They also lacked antibodies of the VCA IgM or IgA classes (<10).

Tumor Patients. Tumor biopsies have been obtained in the course of previous collaborative studies at the ENT Department, Kenyatta National Hospital, by Dr. S. Singh. The biopsies were shipped to Stockholm in dry ice and kept frozen (~70°) until use. Clinical diagnosis, source of tumor biopsy, and EBV serology of the tumor-bearing patients are presented in Table 1. The clinical diagnosis could not be subjected to independent histopathological verification and must be therefore considered as tentative.

EBV Serology. Antibodies to VCA and EBNA were titrated by indirect immunofluorescence (8, 9). Antibodies to EBNA were measured by anticomplement immunofluorescence (18).

Preparation of Antigenic Tumor Extracts. Thawed tumor tissues were homogenized and subsequently sonicated under microscopic control. Further processing was performed as described earlier (28). Sonicates were centrifuged (100,000 x g, 4°, 30 min) and kept frozen (~70°) until use. Protein concentration was measured (4) and adjusted to 50 μg/ml with RPMI 1640. This protein concentration was effective in our previous experiments performed with extracts of EBV genome-carrying cell lines (25, 29).

EBV Genome Detection by Nucleic Acid Hybridization. Cellular DNA was isolated as described by Petterson and Sambrook (16). Filter hybridization between EBV DNA and complementary RNA and the calculation of EBV genome copies per cell were done as described by Andersson-Anvret et al. (2). The exact procedure has been detailed elsewhere (22). Briefly, 10 μg of DNA of each test sample were adsorbed to nitrocellulose filters. 32P-complementary RNA was added, and hybridization was carried out in 6-fold standard saline citrate (1-fold standard saline citrate = 0.15 M NaCl + 0.015 M sodium citrate:50% formamide) at 46° for 4 days. After RNase treatment and repeated washing to remove unhybridized RNA, the filters were dried and counted. DNA from the EBV genome-carrying Raji cell line (5, 17) served as positive control, and DNA from the EBV-negative U-698M cell line (1, 15) was used as negative control. Results were expressed as the average number of viral genome copies per cell.

Leukocyte Migration Inhibition. Direct agarose microdroplet assay

Recessive}

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was carried out according to the method of McCoy et al. (12) with minor modifications (25). Twenty million washed buffy coat cells were mixed with 135 µl nutrient agarose medium, containing an equal volume of 0.4% agarose and 2-fold RPMI 1640 supplemented with 20% heat-inactivated horse serum and antibiotics. Two-µl droplets of the suspension were then placed into migration chambers. After the droplets had solidified, the chambers were filled with RPMI 1640 with or without tumor extract and incubated in a 37°C humidified CO2 atmosphere for 18 to 24 hr. Subsequently, the migration areas were measured, and the MI was calculated according to the formula:

\[ \text{MI} = \frac{\text{Mean of triplicates with antigen (test)}}{\text{Mean of triplicates without antigen (control)}} \]

Statistics. MI < 0.8, i.e., at least 20% inhibition of migration was considered as significant. For group statistics, Student’s t test was used.

RESULTS

Chart 1A shows the LMI tests performed by exposing the leukocytes of healthy donors to extracts of EBV DNA-negative tumors. There was no difference in the response of SP and SN donors to any of the extracts. The extracts “CC” and “MP” inhibited leukocyte migration to a significant degree, but there was no difference between SP and SN donors.

Chart 1B shows the effect of EBV DNA-positive tumor extracts. Fifteen of the 17 biopsies inhibited the migration of leukocytes from SP but not or much less from SN donors. In one of the 2 negative cases (SW), the serology was not in line with what would be expected from an EBV DNA-carrying tumor, and the average EBV genome number was marginal (one). The same was true for the second case (SN) with low serology, marginal EBV genome number (2), and a diagnosis (carcinoma parotis) that would not be expected to associate with EBV (Table 1). The extracts “DM,” “BNK,” and “TM” induced LMI in the SN group as well, but the effect was higher in the SPs. With DM and BNK, the difference was statistically significant.

DISCUSSION

LMI, regarded as an in vitro correlate of delayed hypersensitivity (3, 24), has been useful in detecting cell-mediated immune responses to a wide variety of bacterial, viral, tissue- and tumor-associated antigens (for review, see Ref. 19).

We have been applying the LMI test to EBV-determined antigens. Using the leukocytes of healthy SP donors, purified EBNA evoked strong positive LMI reaction (25, 29). Since the antigens associated with the viral cycle, EA and VCA, were not available in purified form, the use of polyvalent extracts was necessary. Specific responses to them could only be detected in the absence of an anti-EBNA reaction, i.e., in acute mononucleosis or in immunodeficient patients (27).

All previous tests were performed with crude extracts of in vitro-growing cell lines or with antigens purified from them.

In the present study, we used extracts of EBV-carrying and EBV-negative tumors, for the first time, to evoke EBV LMI. We observed a good correlation between the presence of EBV DNA in the tumor and the capacity of biopsy extracts to induce EBV-specific LMI reaction. None of 6 extracts of EBV DNA-negative tumors gave an EBV-specific LMI, whereas 14 of 17 extracts of EBV DNA-positive tumors were capable of doing so, as judged by the differential between the migration of leukocytes from EBV-SP and -SN donors. In one case, the difference was questionable. The 2 biopsy extracts that failed to induce specific LMI contained only marginal quantities of viral DNA (one and 2 genomes per cell), and the patients from whom they were taken had EBV antibody pattern within the normal range.

Two biopsies were not expected to contain viral DNA. One was obtained from a tonsillar carcinoma (CT), the extract-induced EBV-specific LMI, and the patient had an antibody spectrum comparable to those seen in NPC. Since viral DNA or EBNA-positive tumor cells were not detected in numerous biopsies from carcinomas other than NPC (2, 10), the possibility of a misdiagnosis or of mislabeling must be considered. The other

Table 1

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<th>VCA IgA</th>
<th>VCA IgG</th>
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a Expressed as reciprocal of serum antibody titers.

b The DNA content of the tumor biopsy sample was at the borderline of the testable amount (5 µg/filter).
biopsy (SN) was derived from a parotid carcinoma, but the extract prepared therefrom failed to induce an EBV-specific LMI reaction. Salivary glands have been suggested as one habitat for persistent EBV infections (13,14). Indeed, viral DNA and/or EBNA-positive cells have been found in normal parotid gland tissue (30) as well as in parotid tumors (11,20). It could not be determined, however, whether the viral genomes resided in malignant or normal salivary gland cells or in the numerous lymphocytes present in the glands.

There was no clear relationship between the number of EBV genomes per cell and the degree of LMI. In spite of the fact that the amount of EBNA is related to the number of EBV genome copies per cell (6,23), such a correlation could hardly be expected, since all biopsies consist of unknown mixtures of tumor and nontumor cells and since different pieces had to be used for the LMI and the EBV DNA tests.

With these limitations, the good correlation between the presence of EBV DNA and the EBV-specific LMI reaction may be a useful procedure for the identification of EBV genome-carrying tumors. The LMI test is more readily available to immunological laboratories than the nucleic acid hybridization techniques. It certainly may serve for independent confirmation of results obtained in nucleic acid hybridization or EBNA-specific immunofluorescence tests.

REFERENCES


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