Indirect Alkaline Phosphatase Immunoenzymatic Staining for the Detection of Antibodies to Epstein-Barr Virus-Induced Virus Capsid Antigens and Early Antigens

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An indirect alkaline phosphatase immunoenzymatic staining technique was developed for the detection of antibodies against Epstein-Barr virus-induced virus capsid antigens and early antigens in cell smears. The presence of antibodies against Epstein-Barr virus-induced virus capsid antigens and early antigens was revealed by a dark blue staining of cells expressing the antigens. The alkaline phosphatase assay gave a permanent record of the reaction that could be visualized under an ordinary light microscope. The titers obtained with this assay on 91 serum samples were significantly correlated with the titers obtained with an immunofluorescence technique.

Epstein-Barr virus (EBV) is an agent with a worldwide distribution. The primary infection in vivo is associated with several responses ranging from asymptomatic seroconversion to infectious mononucleosis. Moreover, EBV is implicated in the pathogenesis of at least two human malignancies, Burkitt lymphoma and nasopharyngeal carcinoma. After primary infection the virus establishes a latent, persistent infection among B lymphocytes (11). Carriers of the virus may periodically develop a reactivation as indicated by viral excretion and immunological response. In some conditions, such as immunosuppression, malignancy, and pregnancy (1, 5), the reactivated infections are particularly frequent. The evidence of active EBV infections is based usually on the elevations of the immune response to EB viral antigens in the patients.

Serological testing for antibodies to EBV is done routinely in diagnostic laboratories and provides useful information regarding immune status and active EBV infection. Several methodologies are available for the diagnosis of EBV infection, including the test for heterophile antibodies of the Paul Bunnel type, enzyme-linked immunosorbent assays (10), and immunoadherence hemagglutination (7). The indirect immunofluorescence (IF) assay, however, is currently the method most widely used to detect antibodies of different classes against EBV-induced antigens (2).

In recent years, immunoenzymatic staining has been employed as a valid alternative to IF techniques in virological studies. Up to now, the immunoperoxidase technique has been the immunoenzymatic method of choice for the detection and quantitation of viral antibody (3, 4), but the effectiveness of this method can be impaired by the endogenous peroxidase activity commonly found in several mammalian cells.

Alkaline phosphatase has also been applied in enzyme-labeled antibody studies, both in immunohistological methods (9) and in enzyme-linked immunosorbent assay techniques (8). Our study therefore aimed to explore the use of an alkaline phosphatase immunocytochemical staining in the detection of antibodies against EBV-induced viral capsid antigens (VCA) and EBV early antigens (EA). We compared the antibody response to EBV-induced VCA and EA, visualized by immunoalkaline phosphatase assay (IAPHA), with the presence of antibodies to VCA and EA as determined by the IF technique.

MATERIALS AND METHODS

Cells. P3HR1 and Raji lymphoblastoid cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum in the presence of 10 U of penicillin and 50 μg of streptomycin per ml. Cells were passaged routinely twice a week. To assess cell viability, P3HR1 and Raji cells were stained with trypan blue (12).

Antigen preparations. To prepare EBV VCA, the cell suspension from one bottle of P3HR1 cells was centrifuged at 1,000 rpm for 10 min, and the cell pellet was reconstituted in 0.5 ml of phosphate-buffered saline (PBS) (0.15 M, pH 7.4). Drops of cell suspension were applied to a slide; in each spot the cell density was checked microscopically, and the concentration was adjusted, if necessary, to give an almost confluent cell smear. The slides were air dried, fixed in acetone at 4°C for 15 min, and then stored at −20°C. A reference serum (MLZ 78, VCA titer of 1/320; EA negative) was used to test cell preparation, showing 5 to 8% VCA-positive cells. To prepare EBV EA, Raji cells treated with 50 μg of 5-iododeoxyuridine (Sigma Chemical Co.) per ml for 72 h (6) were processed as described above. EA preparations were tested with a reference serum (EJ 77, EA titer of 1/1,280), showing between 5 and 15% EA-positive cells.

Sera. Ninety-one serum samples from normal blood donors, renal transplant recipients, pregnant women, and patients with infectious mononucleosis were tested.

IF assay. Titers of antibodies to VCA and EA were determined by the IF technique. Acetone-fixed cells were incubated with serial dilutions of sera for 45 min at 37°C, washed three times in PBS, and incubated with a 1/20 dilution of fluorescein isothiocyanate-conjugated antibody to human immunoglobulin G (IgG) (Dako) for 45 min at 37°C, washed three times with PBS, and mounted in glycerol-PBS (1:1).

IAPHA. Acetone-fixed cells were treated with serial twofold dilutions of sera at 37°C for 45 min. After three washes in PBS, alkaline phosphatase-labeled goat immunoglobulins to human IgG (KPL Inc.) were added at a dilution of 1/20. Cells were then incubated at 37°C for 45 min; after another...
In our work the presence of antibodies against EBV-induced VCA and EA was determined by an IAPhA. EBV-induced antigens were stained blue since the alkaline phosphatase label was developed with a naphthol salt as a coupling agent and a diazonium salt (Fast Blue RR) as a capture agent, to form an insoluble blue precipitate at the site of the enzyme (Fig. 1). The highest serum dilution which gave a blue staining in VCA- or EA-positive cells was considered the IAPhA titer of the serum sample. Antibody detection by IAPhA was compared with antibody detection as determined by IF.

**Antibodies to VCA.** The determination of antibodies to VCA at IF showed that, of the 91 serum samples examined, 2 were without demonstrable anti-VCA antibody at a dilution of 1/10 and 89 had titers ranging from 1/10 to 1/2,560. With the IAPhA, of the 91 sera examined, 2 proved with titers less than 1/10 and 89 had titers ranging from 1/10 to 1/2,560. Of the 91 serum samples examined 89 proved positive when tested by both assays and 2 proved negative by both procedures. The distribution of values of sera tested by IF and IAPhA is shown in Fig. 2. For the 91 serum samples examined, the correlation coefficient (r) of logarithmic transformations of IF and IAPhA values was 0.95. For the 89 serum samples positive at both assays, the coefficient of correlation was 0.94.

To assess the reproducibility of the IAPhA, 10 sera were selected; 8 sera had anti-VCA IF titers ranging from 1/40 to 1/2,560 and 2 had anti VCA titers less than 1/10. Six replicate samples of each of these sera were tested on different assays to confirm reproducibility. In all 10 sera retested, differences in titers were less than or equal to twofold, and 98% of the observations were within one dilution of the expected antibody value.

**Antibodies to EA.** The IF assay for EBV-induced EA showed that, of the 91 sera examined, 34 had titers less than 1/10 and 57 proved positive with titers ranging from 1/10 to 1/320. With IAPhA, 36 samples had titers less than 1/10 and 55 were positive with titers ranging from 1/10 to 1/320. Of all the 91 serum samples examined, 87 were either positive (54 serum samples) or negative (33 serum samples) by both procedures. The distribution of values of antibody against EBV EA demonstrated by IF and IAPhA is shown in Fig. 3. For the total 91 serum samples examined for antibody against EA, the coefficient of correlation was 0.94, whereas for the 54 samples positive at both assays the coefficient of correlation was 0.90. The reproducibility of the results obtained for antibody against EA by IAPhA was determined in 10 sera as described above for VCA, and the differences in titers were less than or equal to twofold; 97.4% of the observations were within the expected antibody value. To test the specificity of IAPhA, cell smears prepared for VCA and EA were stained by IAPhA with reference human sera negative for EBV-induced antigens; these cell preparations proved uniformly colorless and unstained. Moreover two
sera with elevated serum levels of alkaline phosphatase and two sera with rheumatoid factor were analyzed, and they showed the same titers with both assays.

**DISCUSSION**

In this paper we describe an indirect alkaline phosphatase immunoenzymatic staining for the detection of antibodies to EBV-induced VCA and EA. An immune serum to human IgG labeled with alkaline phosphatase was employed, and the enzyme label was developed with a naphthol salt in the presence of a diazonium salt (Fast Blue RR). Specific IgGs in the human sera examined were visualized by the appearance of a blue precipitate in the cells expressing the corresponding antigens. The serum titers evaluated by the IAPhA were compared with values as determined by the IF technique, the correlation between the values obtained with the two different assays was highly statistically significant. The reproducibility of the IAPhA, both for antibodies to VCA and EA, proved that it is a reliable technique that can be a good alternative to the IF assay. However, compared with IF, IAPhA offers the advantage of giving a permanent record of the reaction that can be easily observed under an ordinary light microscope, and it does not require immediate interpretation since it is dependent on a very stable histochemical reaction. In our assay all of the reagents were commercially available, thus minimizing technical time; the method was simple to perform and did not require costly instruments.

We believe that the similarity of IAPhA to standard procedures in terms of sensitivity and reliability makes this assay an economical addition to current routine serological tests in many diagnostic laboratories and can have further applications in the virological context.

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**LITERATURE CITED**