Detection and Quantification of Type C Viral Proteins in Tissues and Sera with an Enzyme Immunoassay

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The detection and quantification of retroviral proteins in cells, tumors, and sera by an enzyme immunoassay technique (enzyme-linked immunosorbent assay [ELISA]) is described. The ELISA technique has been applied to three viral systems: murine leukemia virus, baboon endogenous virus, and simian sarcoma virus. By detecting 0.1 ng of retroviral antigen, the sensitivity of the ELISA is comparable to that of the competition radioimmunoassay technique.

Several methods have been used in the past for the serological identification and characterization of type C viral proteins (immunoprecipitation, complement fixation, immunodiffusion; cf. 4, 7, 9). The most specific and most sensitive assay system used has been found to be the competition radioimmunoassay (RIA). This assay detects group-specific and interspecies activity of the major viral proteins (15-18). Disadvantages and difficulties of this assay include the need for radioactive isotopes, false-positive results from contaminating proteases, and the limited stability of the radioactively labeled reagents. Recently, the enzyme immunoassay technique has been developed (1, 14, 20). This technique has also proved to be a highly sensitive and specific method for the detection of antigens and antibodies in a variety of systems (10-12, 20). It avoids the biohazards of radioactivity and is simple and cheap. A further advantage is the stability of the coupled reagents. The low cost and simplicity of the enzyme immunoassay technique allow the screening of large numbers of samples.

We describe herein the application of the enzyme-linked immunosorbent assay (ELISA) to the detection and quantification of retroviral proteins in cells, tumors, and sera of three animal systems. In contrast to the competition RIA, the ELISA is a direct binding assay.

We found that in the homologous system the ELISA detected retroviral proteins as well as the RIA. Under appropriate experimental conditions interspecies reactivity was also recognized. The ELISA was superior to the RIA with respect to simplicity and absence of radioactivity.

MATERIALS AND METHODS

Compounds. Horseradish peroxidase grade VI (RZ 3.0), *o*-phenylenediamine-2HCl, Tween 20, and thi-

merosal were purchased from Sigma, Munich; Trasylol was from Bayer, Leverkusen. Human albumin and all commercially available control sera were supplied, if not stated otherwise, by Behring Werke, Marburg (all in West Germany).

Technical supplies. Microtiter plates (M129) were purchased from Dynatech, Plochingen, Germany. For absorbance measurements of ELISA reactions a Beckman spectrophotometer was used initially, and then a Titertek Multiscan (Flow, Bonn) with a 450-nm filter was used. For electrofocusing, a Multiphor (LKB, Munich) was used.

Cell lines and viruses. M7BaEV, M7BaEV-A204, SiSV-71AP1, and initial batches of BaEV p30 and BaEV p30 antisera were the gift of R. Gallo, National Cancer Institute, Bethesda, Md. HF cells were supplied by F. Deinhardt, Munich. The other cell lines and viruses were grown or introduced by our own laboratory (2).

Preparation of antigens. Viral proteins were purified by a two-step electrofocusing procedure in granulated gels, first at a pH range from 3.5 to 10.0 and then at a pH range from 5.0 to 8.0. Their purity was controlled by sodium dodecyl sulfate-gel electrophoresis, and homogeneity was found by gel scan detection.

Preparation of antibodies and purification of immunoglobulin G (IgG). Antibodies were prepared from immunized rabbits and goats. The procedure is detailed for a 4-kg rabbit: 100 μ g of purified viral protein (p30) was boosted at 6-week intervals. The animals were bled weekly from the ear for titer determinations. About 40 ml of blood was collected weekly, starting immediately after the appearance of antibodies. The capacity of the serum to precipitate antigen was followed by double immunodiffusion. The specificity of the antisera was controlled by precipitation of disrupted virus and subsequent gel electrophoretic analysis of precipitated proteins. The activities of the sera were directed almost exclusively against the respective p30's.

IgG was purified by the following procedure: 1 ml of serum was diluted with an equal volume of distilled water, and the IgG was precipitated by the addition of 1.8 ml of saturated NH_4SO_4 to give a 45% NH_4SO_4

solution of 20°C. After 30 min at room temperature, the precipitate was collected by centrifugation (20,000 $\times g$, 10 min, 20°C), dissolved in 0.5 ml of 20 mM sodium phosphate buffer (pH 8.0), and dialyzed against 3 $\times$ 1 liter of the same buffer overnight. The IgG solution was then applied to a 2-ml DE52 cellulose column (5-ml syringe), preequilibrated with 20 mM sodium phosphate buffer (pH 8.0), and eluted with the same buffer. The absorbance at 250 nm (A_{250}) and A_{280} of the flow-through were determined, and fractions with A_{280}/A_{250} ratios of more than 2.5 were combined, concentrated by NH_4SO_4 precipitation, and redissolved in Dulbecco-modified phosphate-buffered saline (PBS) (0.2 g of KCl, 0.2 g of KH_2PO_4 , 8.0 g of NaCl, and 1.92 g of Na_2HPO_4 per liter of water). The purity control of the IgG by immunoelectrophoresis showed a single homogeneous precipitation line.

Coupling of peroxidase to IgG. In essence, a modification of the procedure of Nakane and Kawaoi (13, 14) was followed, which involves blocking of the amino groups of the peroxidase with phenyl isocyanate, introduction of aldehyde groups with sodium periodate, and formation of a Schiff base with the IgG by incubation of the IgG with the activated peroxidase.

The reaction was stopped by addition of sodium borohydride (5 mg/ml; 100 $\mu\text{g}/\text{mg}$ of peroxidase). The enzyme-coupled IgG was separated from the free peroxidase by precipitation with 50% saturated ammonium sulfate. The pellet was resuspended in Dulbecco PBS and dialyzed against the same buffer. A_{280} and A_{403} were measured, and A_{403}/A_{280} ratios of 0.4 to 0.6 were obtained. The peroxidase-coupled IgG preparations were controlled in titrations against purified antigen and found to have good, reproducible serological activity.

ELISA procedure. Microtiter plates were coated with 200 μl of 5 to 10 μg of IgG per ml of 50 mM carbonate-bicarbonate buffer (pH 9.6) per well at 4°C overnight. The next morning the plates were washed vigorously in washing buffer (Dulbecco PBS and 0.1% Tween 20). To block residual absorption sites on the microtiter plates, a second incubation followed with homologous preimmune serum (1% in Dulbecco PBS), 0.1% Tween 20, 5% Trasylol, and 0.1 mM thimerosal for 4 h at room temperature. The plates were washed again with washing buffer. To the antigen of interest in Dulbecco PBS, 0.2% rabbit albumin, 5% Trasylol, 0.1 mM thimerosal, and 0.1% Tween 20 were added, and the plates were incubated at 4°C overnight. A standard concentration curve with purified p30 was included in each test. After the plates were extensively washed, enzyme-coupled IgG in Dulbecco PBS, 5% chicken serum, 5% Trasylol, 0.1 mM thimerosal, and 0.1% Tween 20 were added, and the plates were incubated at 37°C for 1 to 2 h. After the plates were washed again, a 200- μl substrate solution containing 20 mg of *o*-phenylenediamine-2HCl and 0.005% H_2O_2 in 50 ml of substrate buffer (7.74 g of citric acid and 17.93 g of Na_2HPO_4 per liter of water) was added. The color reaction was then followed photometrically. Initially the reactions were stopped with 50 μl of 0.5 N H_2SO_4 , and the absorbances were measured at A_{492} . With the use of a Titertek Multiscan with a 450-nm filter, we followed the reaction at 10-min intervals without stopping. Backgrounds generally ranged from 0.02 to 0.1 A_{450} .

Competition RIA procedure. The double-antibody RIA procedure described by Strand and August (19) was used. Proteins were labeled with ^{125}I according to Greenwood et al. (6). The specific activity obtained was 10³ cpm/ng of protein. The input of ^{125}I -labeled protein was 10 ng in a 50- μl volume of 10 mM PO_4^{3-} (pH 7.5), 140 mM NaCl, 1% chicken serum, 0.1% Triton X-100, and 0.5% Trasylol (RIA buffer).

In the competition assay, increasing amounts of unlabeled antigen (or tumor extracts or sera) were incubated with antibodies at limiting antibody concentrations (1:200-diluted immune serum in 1:20-diluted normal rabbit serum) at 37°C for 2 h. The limiting antibody dilution precipitated 70% of labeled antigen as determined by direct double-antibody RIA. Labeled antigen was then added and incubated at 37°C for 1 additional h. After overnight incubation at 4°C, 50 μl of goat anti-rabbit IgG was added and incubated for 1 h at 37°C and 3 h at 4°C. After dilution with 1 ml of RIA buffer, the immunoprecipitates were collected by centrifugation at 4,000 $\times g$ (20 min, room temperature) and counted in a Berthold and Friesecke gamma counter, type 500.

Cell extraction. Cells were lysed by the addition of 2 volumes of water and extracted with an equal volume of ether. After centrifugations at 2,000 $\times g$ for 1 min to separate the aqueous phase and ether and at 20,000 $\times g$ for 10 min to remove nuclei, mitochondria, and cell debris, the supernatant was dialyzed overnight against Dulbecco PBS. The dialysate was stored at -20°C and used for the assays.

Tissue extraction. Equal amounts of water and tissue (weight/weight) were combined and homogenized with an Ultra-Turrax homogenizer (Jahnke and Kunkel, Freiburg) at full speed and 30-s intervals until the suspension appeared to be homogeneous. Nuclei, mitochondria, and debris were removed by centrifugation at 20,000 $\times g$ (10 min, 4°C). The supernatant was stored at -20°C and used for the assay.

Absorption of IgG. A 6- μg amount of IgG was mixed with increasing concentrations of antigen. The mixture was incubated at 37°C for 1 h and at 4°C overnight. The pH was adjusted to 9.6 with 1 M carbonate-bicarbonate buffer to give a final concentration of 50 mM in a final volume of 600 μl . The solution was spun in microtubes at 45,000 rpm for 45 min (SW50.1 rotor with microadaptors). The immune complexes were removed, and the supernatant (400 μl) was used for the ELISA.

RESULTS

Standardization with purified viral proteins. As a necessary prelude to the meaningful application of the ELISA technique to the detection and quantification of viral antigen in tumor tissue and body fluids, the test system was standardized and optimized with purified viral antigen. We determined the optimal test conditions in three viral systems: murine leukemia-sarcoma viruses (MuLV), simian sarcoma-simian sarcoma-associated viruses (SiSV) (21), and baboon endogenous virus (BaEV) (5). The major viral structural protein p30 was chosen as antigen. p30 can be detected specifically and

sensitively down to quantities of 0.05 to 0.5 ng/250- μ l reaction volume in the three viral systems tested (Fig. 1A). This sensitivity is comparable to that of the RIA.

The antigen binding to the coating IgG on the microtiter plate is linear over a two-log concentration range of antigen and flattens above and below this range to give a sigmoid curve. For better comparison of different tests, maximal absorbance of purified antigen was designated 100%, representing A_{450} (A_{492}) values ranging from 0.7 to 1.7. Fetal calf serum, normal goat and rabbit sera, extracts of uninfected cells of the cell lines in which the viruses were grown, and purified human lipoprotein did not react. The reaction was abolished by pronase digestion, but not by extraction with ether or digestion with nucleases. Preabsorption of the antisera with the homologous p30 also abolished the reaction. MuLV p30 and BaEV p30 were negative in the SiSV system, MuLV p30 and SiSV p30 were negative in the BaEV system, and BaEV p30 and SiSV p30 remained negative in the MuLV system.

The optimal concentrations of the coating IgG and of the peroxidase-coupled IgG were determined as depicted in Fig. 1B and C. All tests were done in antigen excess. By increasing the coating antibody concentration at constant peroxidase-coupled IgG concentrations (Fig. 1B), the maximum binding of the antibody (IgG) to the polystyrol microtiter plate was determined. A plateau was reached at a coating IgG concentration of 5 to 10 ng/ml in all three viral systems. The minimal IgG concentration yielding maximal binding (5 μ g/ml) was used for further testing. By increasing the peroxidase-coupled IgG concentration at a constant coating IgG concentration (Fig. 1C), no plateau was reached even at concentrations of 50 μ g/ml, indicating that the antigen-antibody complexes on the polystyrol plate cannot be saturated with antibody at economical IgG concentrations, but a decrease of slope was observed at concentrations of 10 to 30 μ g/ml. Concentrations in this range (20 μ g/ml) were used for further testing. Under these assay conditions reproducible results were obtained.

Detection of viral protein in cells. In a second series of experiments the detectability by the ELISA method of retroviral p30 in cells was studied. Cytoplasmic extracts were prepared from OS-2 murine cells productive of MuLV, from 71AP1 cells productive of SiSV, and from A204 cells productive of BaEV.

In the extracts from cells productively infected with the homologous virus, retroviral p30 was specifically detected, whereas extracts from uninfected cells remained virtually negative (Fig. 2).

Extracts from cells productively infected with related viruses showed, with anti-MuLV p30 antiserum, group and interspecies reactivities, as would be expected from the known group-specific and interspecies reactivities of the respective p30 proteins: anti-MuLV p30 antiserum detected SiSV p30 with a reaction that was 100% of the homologous reaction. Also, BaEV-producing A204 cells showed some cross-reactivity. The reaction with HF cell extract is due to low-level expression of SiSV p30 by this cell line.

Extracts from tumors and cells producing different viruses (FBJ virus [3], OS-2 and B16 murine endogenous viruses [2, 8], and Rauscher MuLV) of the same virus group (MuLV) showed complete cross-reactivity of their p30 proteins (cf. Fig. 3; data not shown).

In contrast, anti-SiSV p30 and anti-BaEV p30 failed to detect interspecies reactivities in the cell extracts tested (Fig. 2). This lack of interspecies reactivity of the anti-SiSV p30 and anti-BaEV antisera is not yet completely understood, but it has been observed that an inhibition of the reaction occurs if the ratio of total protein to reactive protein exceeds a certain maximum (data not shown).

For the tests with cell extracts, micrograms of cell extract were plotted against percent absorbance. The nanogram amount of p30 per microgram of cell extract can be determined by comparison of an absorbance value on the cell extract concentration curve with an equal absorbance value on the standard concentration curve. A standard concentration curve with purified homologous p30 was included in each experiment.

FIG. 1. Standardization of the ELISA parameters. (A) Concentration curves of purified homologous p30 from MuLV, SiSV, and BaEV. The values are normalized, with 100% corresponding to maximal absorbance in the respective viral system. For the MuLV system maximal absorbance ranged from 1.5 to 1.7 A_{450} ; for the SiSV and BaEV systems it ranged from 0.7 to 1.0 A_{450} . The backgrounds subtracted ranged from 0.02 to 0.1 A_{450} . Antibody concentrations were kept constant at 5 to 10 μ g/ml for the coating antibody and 10 to 20 μ g/ml for enzyme-linked antibody. Symbols: $\circ$, homologous p30 antigen; $\square$ and Δ , heterologous p30 antigens; $\bullet$, fetal calf serum (FCS); $\blacksquare$, normal rabbit serum (NRS); $\blacktriangle$, antigen digested with pronase. (B) Concentration curves of coating antibody. Six concentrations of the coating antibody were tested with eight concentrations of the enzyme-linked antibody. The p30 antigen concentrations were in excess (100 to 200 ng/ml). (C) Concentration curves of the enzyme-linked antibody. Enzyme-linked antibody concentrations are plotted against coating antibody concentrations (reverse plotting of curves from B).

A ELISA: Antigen concentration and specificity

B ELISA: Concentration of enzyme linked antibody

C ELISA: Concentration of coating antibody

FIG. 2. Detection and quantification of viral antigen in cell extracts. Presentation of data is as specified in the legend to Fig. 1. For details see text. Symbols: ○, homologous purified p30 (standard concentration curve). Cell extracts: □, MuLV-infected cells (SC cells); △, SiSV-infected cells (71AP1 cells); ◇, BaEV-infected cells (A204 cells); ●, uninfected murine cells (N-C3H or SC cells); ■, uninfected marmoset cells (HF cells).

Concentration curves and saturation levels of the tests with cell extracts are similar to those obtained in the homologous reactions with purified p30 (Fig. 2).

Controls included tissue culture components such as fetal calf serum, normal goat serum, and preimmune sera. No interference by these components with the reaction could be detected. It is concluded that the ELISA technique detects viral antigens in cells sensitively and specifically.

Detection and quantification of p30 in tumor tissues. In a third series of experiments, the ELISA technique was used for the detection and quantification of viral p30 in tissues, in particular, in tumor tissue.

We examined two solid tumors that were shown to be associated with viruses related to the MuLV-sarcoma complex: B16 mouse melanoma (8) and several radiation- and virus-induced murine osteosarcomas (1a, 3). Normal mouse skin, muscles, and bones were used as control tissues. Tissue extracts were prepared as described in Materials and Methods and were tested under conditions similar to those worked out for extracts from tissue culture cells. MuLV p30 was specifically detected with the ELISA method in the B16 melanoma and in the murine osteosarcomas in quantities ranging from 1.91 mg of p30 per g of B16 melanoma to 0.48 mg of p30 per g of FBJ virus-induced osteosarcoma. As would be expected, minor quantities of p30 were also detected in normal mouse skin (812 ng/g of tissue), muscles, and bones (about 1.5 µg/g of tissue; Fig. 3A).

The quantification of p30 was again done by comparison with the standard p30 concentration curve included in each assay, as described above for tissue culture cells.

As a sensitivity and specificity control, the same tumors were tested with the competition RIA technique (Fig. 3B). As can be seen from Fig. 3A and B, the p30 concentrations measured by the ELISA and RIA methods are similar.

Detection and quantification of p30 in sera. In a fourth series of experiments, we applied the ELISA technique to the detection of viral antigens in sera. A suitable animal system is the Rauscher leukemia of BALB/c mice, which shows significant viremia of MuLV about 3 weeks after infection with the Rauscher strain of MuLV. Sera of BALB/c mice infected with Rauscher MuLV at the age of 4 weeks were tested by the ELISA technique for MuLV p30 3 weeks after infection. Sera of 4- and 7-week-old uninfected mice were tested in parallel. MuLV p30 was specifically detected by the ELISA technique in the sera of the leukemic mice, and, to a lesser extent, in those of the 4- and 7-week-old uninfected mice (Fig. 4A). The p30 concentra-

tion in the sera of the leukemic mice was as high as 333 µg/ml compared with the low concentration in the control sera, which contained less than 150 ng/ml. In a parallel determination with the competition RIA technique, a concentration of 341 µg/ml of serum was measured, a value virtually identical to the ELISA result.

DISCUSSION

We conclude from this study that in the homologous system the ELISA technique can quantitatively detect retroviral proteins in cells, tumors, and sera. Although the ELISA technique has been used for the detection of other viral antigens (1, 10, 12, 20) and retroviral antibodies (11), its applicability to the detection and quantification of retroviral proteins has not been described yet. This method allows the simple and fast detection, and the determination of the concentration, of retroviral proteins even in tissues and sera in which viral presence is in question, e.g., in chemically or radiation induced tumors or in spontaneous tumors. The spontaneous mouse melanoma B16 (8) and the radiation-induced osteosarcoma (1a) used in this study are examples of this applicability of the ELISA.

Interspecies reactivity has been detected with the ELISA in the reactions with anti-MuLV p30 antiserum. The failure of anti-MuLV p30 antiserum to recognize interspecies determinants on the purified p30 proteins is attributed to the fact that highly purified p30 proteins may lose interspecies determinants during or after the purification process. The failure of the anti-BaEV p30 and SiSV p30 antisera to detect interspecies reactivity in the ELISA system is not yet fully understood. It is the object of present research to determine how extensively the ELISA can be used for the detection of interspecies reactivity.

Since the ELISA is a binding assay and no competition is involved, we would expect, however, a somewhat broader specificity of the ELISA than of a competition assay. This expectation is supported by the maximal reaction (100% of the homologous reaction) with anti-MuLV p30 in recognizing interspecies determinants in SiSV p30-containing cell extracts. In detecting 0.1 ng of retroviral antigen and less per 200-µl reaction volume, the sensitivity of the ELISA technique is comparable to that of the RIA. The ELISA technique is superior to the RIA with respect to simplicity, absence of radioactivity, and longer stability of coupled reagents. It avoids false-positive results from proteases, which cause confusion in the RIA system if protease inhibitors are not included. Proteases would yield negative results in the ELISA system. The absence of radioactivity in addition to

FIG. 3. Detection and quantification of viral antigen in tumor tissue by ELISA (A) versus RIA (B). For ELISA, presentation of data is as detailed in the legend to Fig. 1. The amount of antigen in the cell extract was determined as equivalent to the amount of purified p30 necessary to achieve 50% absorbance on the standard concentration curve. In the RIA the tissue extracts were used to compete with the iodinated p30. The values are normalized to 100% binding representing 70% of the radioactivity and 0% binding representing a 5% background.

FIG. 4. Detection and quantification of viral antigen in sera by ELISA (A) versus RIA (B). Presentation of data is as in Fig. 3 and detailed (for ELISA) in the legend to Fig. 1. Pooled sera of 10 infected mice injected with Rauscher MuLV-infected BALB/c spleen homogenates and of 10 uninfected control animals were tested. Infected and uninfected animals were examined on the day of injection (at the age of 4 weeks) and 3 weeks after injection (at the age of 7 weeks) when overt leukemia became apparent in the infected animals.

the simplicity, low cost, and low material requirement of the ELISA technique make the ELISA more generally applicable than the RIA.

In the present paper the procedure has been described for the viral structural protein p30. Similar results have been obtained for the detection and quantification of type C viral glycoproteins (L. H. Schetters, R. Hehlmann, and V. Erfle, manuscript in preparation).

We suggest that the ELISA system is a suitable system for studies on the association of type C viral structures with leukemia and other mesenchymal neoplasias in animals. How widely the ELISA can be applied to the search for interspecies reactivities in humans will be the object of future research.

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