Quantification of Infection and Neutralization of Murine Leukaemia Virus by a Microtitre Enzyme-linked Immunosorbent Assay

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SUMMARY

We have developed an enzyme-linked immunosorbent assay for murine leukaemia virus using methanol-fixed cells as antigen. Specific antisera clearly recognized viral antigens within cells adherent to the microtitre plates. This test was used to quantify infection, to monitor virus multiplication and to demonstrate virus neutralization by antisera. The ELISA was as sensitive as, and faster and easier to perform than, the XC plaque assay and could even quantify large amounts of virus. It can easily be adapted for assaying other viruses.

Test systems to detect infectious virus are necessary not only to quantify virus particles but also to measure the neutralizing effects of antibodies or to search for viral mutants. The first tests used were plaque tests based on the cytopathic effect of the viral infection in monolayer cultures of host cells (Dulbecco, 1952). Since most of the retroviruses do not lyse their host cells, a secondary effect of the viral infection has to be used in quantitative assays. Bassinet al. (1971) observed that a revertant subline of 3T3 cells (S+L-), originally transformed by Moloney murine sarcoma virus, re-transformed when superinfected by murine leukaemia virus (MuLV). The transformed cells detach from the culture dishes and leave plaques in monolayers. Rowe et al. (1970) used the cell fusion activity of the retroviruses to develop the effective and most widely used XC plaque assay. This plaque test requires about 9 days and is carried out in several steps that include two different cell lines. Nexo (1977) detected centres of viral infection on a cell monolayer as plaques by immunohistochemical staining. This method avoids the use of a second cell line.

Here we report a simple microtitre enzyme-linked immunosorbent assay (ELISA) that measures viral antigens in infected cells. We show that this test is suited for quantification and discrimination of infectious viruses.

In initial experiments, we examined whether viral polypeptides can be detected in fixed cells by an ELISA. A test system using purified polypeptides as antigen was modified (Bayer et al., 1984). Cells of different lines were detached from their culture bottles by incubation with 1.25 g/l EDTA and 1.25 g/l trypsin in phosphate-buffered saline (PBS; pH 7.2). Fifty-thousand cells per well were seeded into 96-well microtitre plates (Falcon) in 150 µl Dulbecco's modified Eagle's medium (DMEM; Dulbecco & Freeman, 1959) supplemented with 10% heat-inactivated foetal bovine serum (FBS). The next day the culture medium was removed, the wells were washed with 200 µl methanol and the cells were fixed in 200 µl methanol for 20 min at room temperature. The plates coated with cells were dried for at least 2 h or overnight at room temperature. To block non-specific binding of antibodies, the wells were incubated with 100 µl PBS containing 0.05% Tween 20 and 10% FBS (incubation buffer) for 15 min at 37 °C. The fluid was then removed and 100 µl of antiserum to viral structural polypeptides, diluted in incubation buffer, was added for 1 h at 37 °C. The wells were then washed three times with 200 µl PBS containing 0.05% Tween 20 (washing buffer) and incubated for 30 min at 37 °C with 100 µl peroxidase-conjugated second antibodies (Medac, Hamburg, F.R.G.) diluted 1/1000 in washing buffer. The wells were washed again and 100 µl citrate-phosphate buffer pH 5-0, containing 50
Fig. 1. Detection of viral antigen in cells by ELISA. Reactivities of a normal rabbit serum (■) and rabbit antisera against the F-MuLV proteins gp70 (▲) and p30 (●) with (a) MuLV-infected FEM, (b) F-MuLV CI 57, (c) STU 3K cells, (d) uninfected NIH 3T3 cells, (e) XC and (f) CRFK cells. Cells had been seeded in 96-well microtitre plates and fixed with methanol as described in the text.

mm-citric acid and 100 mm-Na₃HPO₄, supplemented with 0.4 g/l o-phenylenediamine and 0.003 % H₂O₂ were added as substrate for the peroxidase. The reaction was stopped after 5 min by adding 100 μl 0.65 M-H₂SO₄ and the absorbance at 492 nm was measured in the wells by a Titertek Multiskan photometer (Flow Laboratories).

Rabbit antisera against the core polypeptide p30 (Hunsmann et al., 1981) and against the glycopolypeptide gp70 of the envelope (Bayer et al., 1984) of the Friend MuLV (F-MuLV) were used. The antigens had been prepared from the supernatant of F-MuLV-producing Eveline cells (Seifert et al., 1975). We examined Friend Eveline monolayer cells (FEM), NIH 3T3 cells that were infected with a molecularly cloned Friend virus (F-MuLV CI 57; Oliff et al., 1980), and fibroblasts from STU mice (STU 3K) that had an activated endogenous MuLV (G. Pauli, Universität Berlin, Berlin, F.R.G., unpublished). All three cell types formed syncytia when mixed with XC cells. Uninfected NIH 3T3 cells as well as XC cells and cat fibroblasts (CRFK; Crandell et al., 1973) were used as controls in the ELISA (Fig. 1). Because the antisera had been prepared against F-MuLV isolated from FEM cells, the highest ELISA values were observed with the FEM cells. The antiserum to the viral core polypeptide reacted strongly with FEM and F-MuLV CI 57 cells and weakly with STU 3K cells. The antiserum to the viral glycopolypeptide (gp70) bound well to FEM, weakly to F-MuLV CI 57 and only marginally to STU 3K cells. Neither antiserum bound to uninfected control cells. Likewise, normal rabbit serum did not bind to any of the six cell lines. The observed differences in the reaction of the antisera to p30 and gp70 with the three MuLV-infected cell lines agreed with the immunological classification of F-MuLV antigens, namely that gp70 contains mainly type-specific and p30 carries group- and interspecies-specific determinants (Schäfer et al., 1969; Hunsmann et al., 1974). Furthermore, detection was specific for different isolates of the same virus. These results showed that viral antigens can be identified in fixed cells by ELISA using specific antisera.

We also investigated whether virus multiplication could be monitored using this approach. For this, NIH 3T3 cells were seeded into microtitre plates in 150 μl DMEM containing 5% FBS and 2 μg/ml Polybrene. Nine parallel tests were made. The culture medium was removed the following day, 80 XC p.f.u. of F-MuLV CI 57 in 50 μl DMEM containing 5% FBS were incubated for 90 min at 37 °C. Then 100 μl of culture medium was added. The cells in one of the parallel tests were fixed as described above at successive 24 h intervals. The culture medium was changed at days 3 and 6. The ELISA was performed after the 8th day using the anti-p30 serum.
Fig. 2. Infection of NIH 3T3 cells with F-MuLV. (a) 500, (b) 1000, (c) 2000 and (d) 4000 NIH 3T3 cells were seeded per well in microtitre plates and infected with F-MuLV on day zero. Control cells remained uninfected. The replication of the virus was monitored daily following seeding by fixing the cells in one of the nine parallel test plates and determining the amount of virus by ELISA using a 1/300 diluted rabbit antiserum raised against the viral core polypeptide p30.

Fig. 3. Sensitivity of the ELISA infection assay. Two-thousand NIH 3T3 cells were seeded per well in microtitre plates and infected with increasing amounts (XC p.f.u.) of F-MuLV isolated from F-MuLV CI 57 cells or from spleens of leukaemic mice. Anti-p30 serum diluted 1/300 was used in the ELISA.

diluted 1/300. The results obtained in different assays starting with 500, 1000, 2000 or 4000 cells/well are shown in Fig. 2(a) to (d) respectively. Viral multiplication can clearly be followed. Depending on the number of cells, p30 was identified 2 to 3 days after infection. Maximum values were reached after 5 to 8 days. The maximum detectable was due to the limited space in the microtitre wells. The fluctuations in the maximum value were due to the changing of the culture medium on the 6th day after infection. In subsequent tests, 2000 cells were seeded per well and cells were fixed at day 5 after infection. This ELISA infection assay required 3 days less than the XC plaque test.

The sensitivity of this ELISA test was compared to the XC plaque assay using increasing virus concentrations for the infection. The XC plaque test was performed as described by Rowe et al. (1970) and one to about 100 plaques could be distinguished on 6 cm culture dishes. A nearly linear correlation was observed between the \( A_{492} \) in the ELISA test and the logarithm of the p.f.u. used for infection (Fig. 3). The ELISA test was not saturated at 2000 p.f.u., 50 p.f.u. resulted in a strong signal, and the value for 5 p.f.u. was two to three times over background. The ELISA test therefore had a broader range of sensitivity than, but was almost as sensitive as, the XC plaque test.

We further checked whether neutralization of the viral infectivity by antisera can be measured by the ELISA. Antibodies to gp70 block the infectivity of F-MuLV \textit{in vivo} and \textit{in vitro} (Hunsmann et al., 1974, 1981). In the experiment, the neutralization of 80 p.f.u. of F-MuLV was performed by mixing the virus immediately before its addition to the cells with an antiserum against the viral glycopolypeptide gp70. The antiserum was diluted in preimmune rabbit serum. Viral infectivity was completely neutralized up to an antiserum dilution of 1/125 (Fig. 4).

The results reported here showed that a microtitre ELISA is suitable for measurement of retrovirus antigens in infected cells. The test can be used to characterize the infectious virus by specific antisera. It is useful also as an infection, multiplication and neutralization assay. The sensitivity corresponds to that of the XC plaque test and even large amounts of virus resulted in
Fig. 4. Virus neutralization by antibodies to gp70. NIH 3T3 cells were infected with F-MuLV (80 p.f.u.) which had been incubated with increasing dilutions of an antiserum to the viral glycopolypeptide gp70. The ELISA was performed as described in the text.

quantitative signals. Recently, we have used this ELISA test to measure the infectivity of feline leukaemia virus, and obtained results similar to those described here for F-MuLV. Because some retroviruses cannot be detected in plaque tests, this ELISA might easily be adapted to investigate them as it needs only a susceptible cell line and a specific antiserum. Since specific antiseras distinguish between different viruses, the infection of MuLV-producing cells with a second leukaemia virus can be measured. Because the microtitre test is easy, rapid and can use automatic ELISA equipment, it represents a useful alternative to the standard plaque assay.

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REFERENCES

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