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Contained Indirect Viable-Cell Membrane Immunofluorescence Microassay for Surface Antigen Analysis of Cells Infected with Hazardous Viruses

MILES W. CLOYD* AND DARELL D. BIGNER

Departments of Pathology and Surgery, Duke University Medical Center, Durham, North Carolina 27710*

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A microtechnique for an indirect viable-cell membrane immunofluorescence titration assay was developed using Friend murine leukemia virus (F-MuLV)-producing cells and monospecific rabbit antisera to F-MuLV structural antigens. The assay was sensitive and displayed little variation within or between assays. Since moderate-risk tumor viruses, such as recently discovered primate oncornaviruses or feline leukemia virus (FeLV), may be hazardous to laboratory personnel, the assay was adapted for containment of cells infected with such viruses. Cells producing gibbon ape lymphoma virus or FeLV were grown in class III containment cabinets and transferred in sealed flasks to a class II laminar-flow cabinet, where the assay was performed. This micromethod not only conserved reagents but also minimized the numbers of moderate-risk tumor virus-infected cells handled at one time. Centrifugation was contained using custom-made devices shown to form a gas-tight seal over microtiter plates. Interspecies reactivity of monospecific rabbit antisera against F-MuLV structural antigen gp71, but not against p12, was demonstrated for surface antigens on FeLV-producing cells.

Serological methods, such as complement-mediated cytotoxicity (3), radioimmunoassay (S. E. Harwood, D. D. Bigner, W. Wechsler, and E. D. Day, J. Natl. Cancer Inst., in press), immunoferritin electron microscopy (24), immunoenzyme techniques (1), and immunofluorescence, are sensitive and practical methods of molecular analysis with which to explore expression of cell surface antigens. Each method in turn has its own inherent advantages and disadvantages when considered for any particular problem. The fluorochrome-labeled antibody technique, originally developed by Coons and co-workers (4), has been widely applied for detection of antigens in tissues and cells, and has been refined into a number of methodologies (2, 5, 13, 14, 16, 17, 27). This technique offers the advantage of direct visualization of bound antibody on the surfaces of viable cells, and is generally more rapid and quantifiable, although less detailed, than immunoelectron microscopy techniques. As a basic and screening method for characterization of reagent antisera and surface antigens on normal and malignant cells, we developed, and report herein, a micromodification of an indirect viable-cell immunofluorescent assay, which is a sensitive, reliable, rapid, and economical method for detecting antbodies bound to cell surface antigens. The assay was easily contained for use with cells replicating moderate-risk tumor viruses.

MATERIALS AND METHODS

Indirect microimmunofluorescence assay. The assay procedure utilized complete Eagle calcium-free Spinner culture medium containing 10% fetal calf serum for all washings and suspensions of cells, and Eppendorf microliter pipettes for dispensing of cell suspensions and antisera dilution. Cells were scraped off the bottom of petri dishes and dispersed into single-cell suspensions through a 14-gauge needle and syringe. Into individual wells of a microtiter plate (disposable polystyrene 96 U-well microtitration plates, Cooke Laboratory Products), 50 μl of primary rabbit antiserum (primary sera refers to the test sera initially applied to the cells as opposed to the secondary labeled antiserum, which is subsequently applied and has specific activity for immunoglobulin G of the primary sera) or preimmune serum was pipetted at serial $\log_2$ dilutions, followed by 25 μl of target-cell suspension, at a concentration of 2×10^7 cells/ml. Precut acetate sealing tape (Cooke Laboratory Products) was placed over the well openings, and the serological reaction was allowed to proceed for 20 min, with shaking every 5 min, at room temperature. The plate was then centrifuged at 900 rpm ($180 \times g$) for 5 min at 18°C in an IEC PR-6000 centrifuge with a no. 276 head. The supernatant fluids from each well were removed with Pasteur pipettes and discarded into a 0.5 to 1.0% providone-iodine solution (Betadine, Purdue

Frederick Co., Norwalk, Conn.), which was later autoclaved. Three drops of medium were added to each well, and the cells were resuspended by shaking and then recentrifuged. After two more identical washes, the cells were resuspended in 50 μl of secondary antiserum, fluorescein-conjugated immunoglobulin G of goat anti-rabbit immunoglobulin G (1:40 dilution resulting in 0.2 mg of total protein per ml, fluorescence to protein ratio = 4.3) (Meloy Laboratories, Inc., Springfield, Va.), previously preabsorbed with rat liver powder. Incubation and washing was the same as with the primary sera. The final cell pellets were each resuspended in 1 drop of isotonic sucrose solution (8.5%) and mounted under cover slips on microscope slides. Between 100 and 200 cells per serum dilution were scored as exhibiting membrane fluorescence or not, using a Zeiss Universal microscope and dark-field ultraviolet illumination with BG 38 exciter, KP500 interference, and 47 barrier filters. Dead cells, which fluoresced diffusely (17), were not scored, and there were usually no more than 5 to 10% dead cells. The fewest dead cells were obtained by washing with medium containing 10% fetal calf serum, rather than phosphate-buffered saline or medium without serum. Clumping of cells, which varied with different cell lines, was minimized by using Spinner medium and low-speed centrifugations. A fluorescence index (FI) per antiserum dilution was calculated as follows: $FI = 100 \times (A - B)/A$ where A is the percentage of nonfluorescing cells with preimmune serum and B is the percentage of nonfluorescing cells with antiserum (13).

This technique proved useful in detecting Friend murine leukemia virus (F-MuLV) P30 on surfaces of murine cells (7), and has been used extensively in studying the expressions of the various antigenic determinants of gp71 on murine and heterologous species cells (M. W. Cloyd, D. P. Bolognesi, and D. D. Bigner, submitted for publication).

To determine the reproducibility of the assay from day to day and within the assay on 1 day, titrations of pooled anti-Friend gp71 antisera were performed on F-MuLV-producing Eveline cells (23) on different days and in triplicate. The mean reciprocal $\log_2$ 50% titer of the antiserum against Eveline cells was 7.29 ± 0.14, giving a coefficient of variation of 1.29% for 11 separate determinations. Counting reliability was calculated by scoring cells in different fields on one slide 10 times each, up to a total of 200 cells scored. Mean percentages of fluorescing cells for 100 and 200 total cells counted were 67.1 ± 2.7% and 65.8 ± 2.1%, respectively (antiserum dilution, 1:128), with no significant variation noted ($\chi^2 = 0.434$; $0.5 < P < 0.6$ that there was a significant difference).

RESULTS

Application of the immunofluorescence assay to cells producing moderate-risk tumor viruses. Since the biological properties and human risks of certain viral isolates, such as those from nonhuman primates or feline leukemia virus (FeLV), are not completely known, use of adequate containment equipment is indicated with these moderate-risk tumor viruses to keep personnel exposure to a minimum (18). This immunofluorescence assay was consequently modified for use with cells producing such viruses. Feline leukemia (F-422) (20) or gibbon ape lymphoma (GAL) cells (12), replicating type C viruses, were grown in sealed suspension culture vessels enclosed in class III gastight glove boxes, and the assay was performed in a vertical laminar-flow class II hood (Baker Co., Inc., Sanford, Maine) in a containment laboratory designed to handle low, moderate- and high-risk tumor viruses (Module I, Animal and Laboratory Isolation Facility, Duke Comprehensive Cancer Center). All centrifugation was contained to eliminate aerosol formation (21) by using sealed shields (IEC no. 1124) for centrifuge tubes and microtiter plate centrifuge carriers (Cooke Laboratory Products, Alexandria, Va.), modified by the Duke University Surgical Instrument Shop to be sealable (Fig. 1). This modified carrier did not leak Freon II vapor from sealed microtiter plates within, before or after repeated steam sterilizations at 22 lb/in^2 steam pressure (250°F [121°C]) for 30 min, using a halogen leak detector capable of detecting a Freon concentration of less than 10^{-9} (TIF Instruments Inc., Miami, Fla.). At the completion of the assay, all glassware and instruments were removed from the hood under a solution of Betadine and steam autoclaved.

Using this immunofluorescence assay, oncornavirus gp71 interspecies antigenic determinants were detected on the surfaces of (FeLV) F-422 and GAL cells. As an example, Fig. 2 illustrates the relatively large extent of reactivity for F-422 cells of monospecific rabbit antiserum against F-MuLV gp71. Antisera against F-MuLV p12 and avian P27 were both nonreactive (Fig. 2). F-422 cells, which are of thymic origin, predominantly displayed capping of membrane fluorescence with gp71 antiserum (Fig. 2 inset). Absorption of gp71 antiserum (diluted 1:10) with an equal volume of packed avian myeloblasts did not remove any reactivity for F-422 (FeLV) cells, whereas similar absorptions with F-MuLV producing Eveline and F-422 cells removed all the immunofluorescence reactivity (data not shown).

DISCUSSION

An indirect viable-cell membrane immunofluorescence microassay is described which, because of its reproducibility, sensitivity, and rapidity, is a useful technique for screening and characterizing reagent antisera and cell surface antigens. The assay procedure is equally applicable to immunoelectron microscopy.

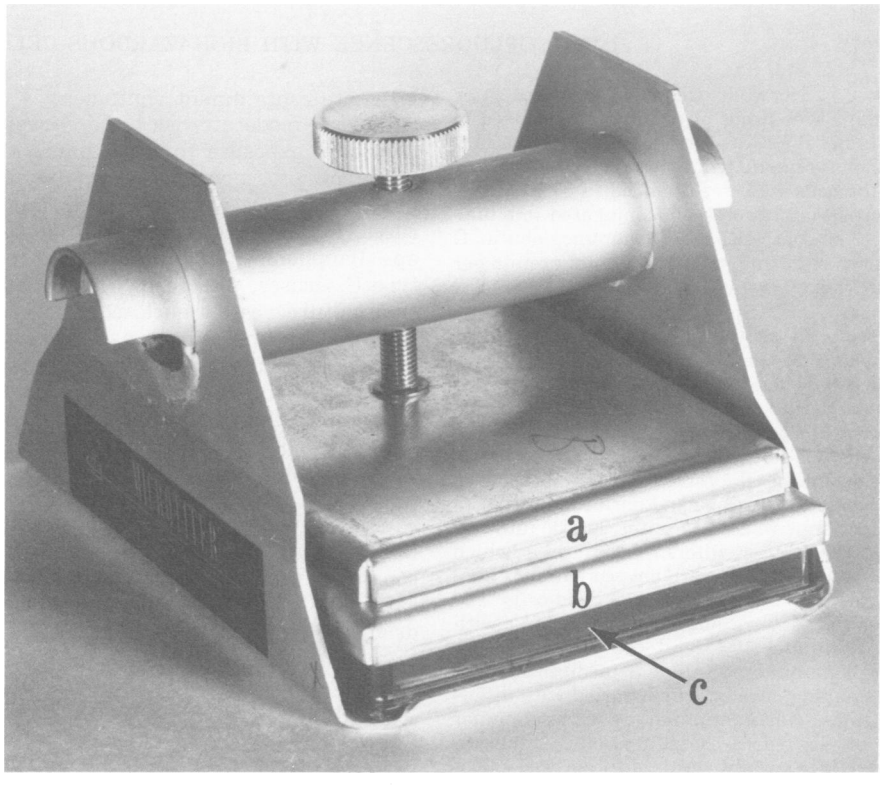

FIG. 1. *Microtiter plate centrifuge carrier, modified for use with cells infected with moderate-risk viruses. A screw-driven plate (a) was added, which exerts pressure on a silicone-lined overlay plate (b) covering the well openings of a microtiter plate (c).*

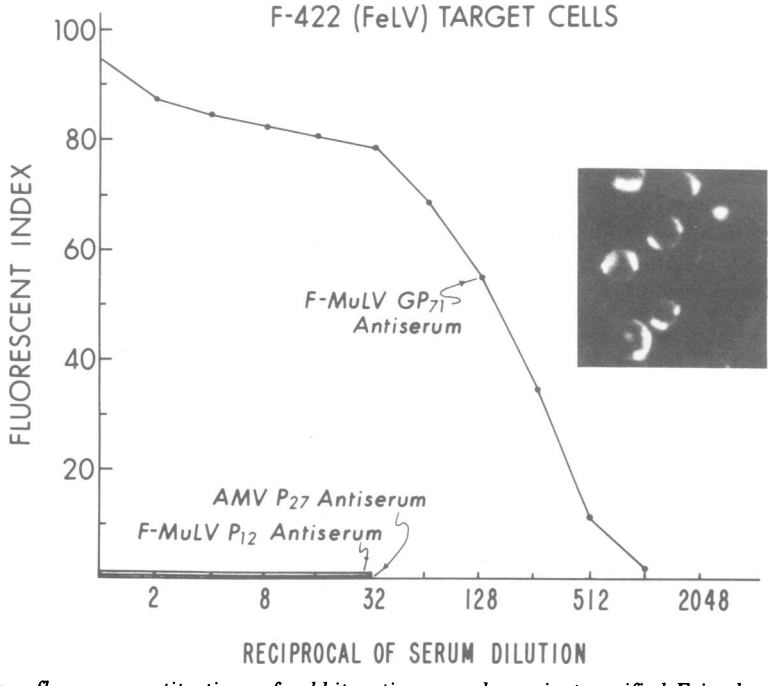

FIG. 2. *Immunofluorescence titrations of rabbit antisera made against purified Friend gp71, p12, and avian myeloblastosis virus p27, respectively, on (FeLV) F-422 target cells. The percentage of cells fluorescing with antiserum above that with preimmune serum is plotted as a fluorescent index for each serum dilution. (Inset) Membrane immunofluorescence patterns obtained with (FeLV) F-422 cells and Friend gp71 antiserum. × 400*

Occasionally, diverse serological results are obtained by using different techniques (15, 19), and more than one serological approach for cellular antigen characterization is needed. MuLV gp71 interspecies cross-reactions have been found by complement fixation with FeLV, feline sarcoma virus, rat leukemia virus, and hamster leukemia virus, but not with RD-114, pig (PLCP), simian sarcoma virus, GAL virus, or avian viruses (9), and with FeLV and feline sarcoma virus, but not primate viruses, using competition radioimmunoassay (25/26). Using chromium release and immunoelectron microscopy techniques, F-MuLV gp71 interspecies cross-reactions similarly appeared to be restricted to those between cells producing oncornaviruses of small rodents and ecotropic FeLV only (10, 23). However, in other radioimmunoassay studies, minor MuLV gp71 interspecies relationships were also established with pig, simian sarcoma virus, and GAL viruses (W. Schäfer and D. P. Bolognesi, Contemp. Top. Immunobiol., in press) and, although no MuLV gp71 cross-reaction to FeLV-producing cells was previously found with cellular immunofluorescence (11), our study using viable-cell membrane immunofluorescence detected MuLV gp71 cross-reactions with both GAL virus- and FeLV-producing cells. Immunofluorescence is thus another useful technique for unraveling the serological complexities of mammalian virus structural antigens.

In addition, work involving biological agents that have unknown or potential pathogenicity for man must be controlled or contained to prevent environmental contamination. Recommendations set forth by the U.S. Department of Health, Education, and Welfare and the National Cancer Institute (8, 18) suggest a minimum of class II containment of moderate-risk agents, such as FeLV or primate oncornaviruses, whereby any environmental exposure occurs in vertical laminar-flow biological safety cabinets. Also, aerosol-producing procedures, such as centrifugation, are of primary concern with any work involving infectious agents, and should be contained (6, 21). Centrifugation can be contained by either enclosing the centrifuge beneath class III safety cabinets of class II laminar-flow cabinets, or by containing the material to be centrifuged. We have chosen the latter because it is less expensive in terms of equipment. Moreover, procedures can be carried out much more efficiently outside containment cabinets. This modified immunofluorescence assay thus meets the recommended safety standards by having all manipulations, except centrifugation, performed in a laminar-flow class II hood, and by using sealed carriers during centrifugations. The only exposure of the cells to the environment is at the end of the procedure when the cells are examined under cover slips in the microscope. After the numerous washings during the assay, few virions are likely to remain to escape from under the edge of a cover slip. However, if complete containment is desired on the microscope, a microscope enclosed in a cabinet could be used, or the cover slips could be sealed at their edges with stopcock grease. Although we have only used the method for tumor virus-infected cells, it should be equally useful for surface antigen analysis of cells infected with hazardous nononcogenic viruses.

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