Specific Immunofluorescence Test for Detection of Herpesvirus Antigen in Cells of the Lucke Renal Adenocarcinoma[1] (36272)

STEVEN M. PAUL,[2] MERLE MIZELL, BRANCH CRAIGE, JULIA BLAZEK, AND MARGARET SKINNER

The Chapman H. Hyams III Laboratory of Tumor Cell Biology, Tulane University, New Orleans, Louisiana 70118

The Lucké renal adenocarcinoma of the common leopard frog (*Rana pipiens*) is particularly suitable for studies in oncogenic virology [see recent review (1)]. Its suspected herpesvirus etiology (2) not only parallels that of other animals (3–5), including man (6), but high yields of virus ($> 10^{11}$ virus particles/g of tumor) can be recovered after several months of low temperature treatment (7). This fact differentiates the Lucké tumor from many other tumor–virus systems. In the above mentioned systems for example, electron microscopy reveals virus only in cells cultured from the malignancies and rarely in the primary tumors themselves. Similarly Lucké tumors display two distinct morphological states: the summer, nonviral, noninclusion state and the winter (virus-containing) state exhibiting acidophilic Cowdry type A intranuclear inclusions, marginated chromatin, and a paucity of mitotic activity (8). Up to now, detection of virus in the Lucké tumor was accomplished only by electron microscopy. However, with the availability of purified virus preparations, immunological techniques became feasible. The present study reports the development of a fluorescent antibody technique for detecting viral antigen(s) in the winter state of the Lucké tumor.

Materials and Methods. Preparation of antigen. Viral fractions used for immunization were prepared by first homogenizing virus containing winter tumors in a Teflon glass homogenizer using 0.25 *M* sucrose–TKM (0.05 *M* Tris, 0.025 *M* KCl, 0.005 *M* $MgCl_2$) as a suspending medium. Differential centrifugation into "nuclear," "mitochondrial," and "mitochondrial supernatant" fractions was followed by zonal centrifugation of the "mitochondrial fraction" on a 10 to 60% sucrose gradient cushioned with potassium citrate. Twenty-four individual fractions were collected and each was centrifuged at 80,000*g* to give pellets which were suspended in 2 ml of phosphate buffered saline (PBS). Each of these viral suspensions were evaluated by a semiquantitative negative staining method using 1:5 dilutions of samples in 2% potassium phosphotungstate (pH 4.5), containing 0.1% bovine serum albumin. The diluted sample was applied to 200 mesh carbon/Formvar coated ionized copper grids and viewed in the Siemens Elmiscope 1A (7). We thank I. Toplin and the John L. Smith Memorial for Cancer Research Chas. Pfizer and Co. Inc., Maywood, New Jersey for the above virus fractionation. For additional details see Ref. (7). From these 24 fractions two adjacent fractions were chosen on the basis of purity and high content of virus ($>5 \times 10^{10}$ virus particles/ml) for the subsequent immunization.

Antisera. An adult rabbit was injected with a total of 0.85 mg of protein with complete Freund's adjuvant. Six injections were spread over a number of weeks, using both intradermal and subcutaneous routes. The animal was subsequently boostered and bled out.

Absorption of antisera. The immune rabbit serum (IRS) was absorbed 5 times with normal frog tissue homogenates (kidney, liv-

[1] These investigations were supported by U.S. Public Health Service Grant CA-011901 from the National Cancer Institute, USPHS - 5 SO1-FRR-05377-11 and Damon Runyan Grant DRG-1055.

[2] Present address: First year medical class, Tulane Medical Center. Address reprint requests to M. Mizell.

er, and spleen) and finally with a nonviral summer tumor homogenate. Incubation of antisera and homogenates was carried out at 37° for 1 hr and then left overnight at 4°.

Fluorescein-conjugated goat antirabbit serum (Hyland Laboratories, Lot No. 2232MOOZAI) was absorbed twice with rehydrated mouse liver powder and once with a normal frog kidney homogenate.

Immunological techniques. Immunofluorescence and Ouchterlony immunodiffusion. The activity of the heterologous antiserum was first checked by standard Ouchterlony immunodiffusion techniques. In all other studies the indirect immunofluorescence technique was employed. Frozen sections cut at 6 μ on an International cryostat were fixed in cold acetone for 15 to 30 min. Primary incubation with IRS was carried out for 20 min at room temperature followed by three 5 min washings in PBS (pH 7.2). Fluorescein conjugated goat antirabbit serum was then applied for an additional 20 min. After rewashing in PBS, slides were mounted and viewed under a Leitz fluorescence microscope. An Osram HBO 200 W bulb was used as a light source. Primary filters were BG38 and BG12 with a yellow Kodak Wratten 12 secondary filter. Occasionally counterstaining with Evan's blue (1:5000) was employed to minimize the normal tissue autofluorescence (9).

Controls. Controls consisted of substituting preimmunization serum for the immune rabbit serum and nonconjugated goat antirabbit serum (Hyland Laboratories, Lot No. 8232MOO2AI) for the conjugated serum. Tissue controls consisted of normal frog kidney and summer (nonviral) tumor sections; normal rat kidney was used as an additional control.

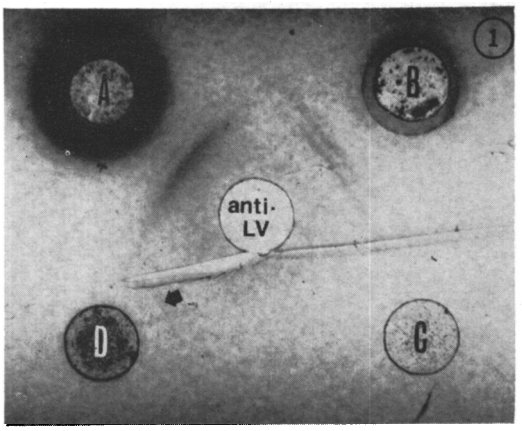

FIG. 1. Results of immunodiffusion tests with rabbit anti-Lucké herpesvirus serum (anti-LV) and: (A) herpesvirus from zonal fraction of winter tumor (virus recharged at 24 hr); (B) herpesvirus from zonal fraction of another winter tumor; (C) normal frog kidney homogenate (diluted 1:10); (D) summer tumor homogenate (diluted 1:10). Note the two precipitation lines between both (A) and (B); the lack of preciptiation between (C); and the single faint line of precipitation (arrow) between (D). (The horizontal tear in the agar occurred during drying of agar before staining).

Results. Figure 1 demonstrates the antisera's strong activity against two different virus fractions similar to those used for immunization. Sera absorbed with normal frog

TABLE I. Indirect Immunofluorescence Test as Applied to Eight Primary Lucké Renal Adenocarcinomas.[a]

Tumor	Days at 7.5°	Immuno-fluorescence	Virus (EM)	Comments
WCI0-70 BCI	132	+	+	CF, CM, LF
VR9-70 UCI	91	+	+	NF, NM, CF, LF
VR9-70 TCI	115	+	+	NF, NM, CF
VR10-69 ACI	140	+	+	NM, CF, CM
VR10-69 KCI	100	+	+	CF, CM
VR3-70 E	22	—	—	—
VR3-69 L	0	—	—	—
VR5-70 E	0	—	—	—

[a] NF, nuclear fluorescence; NM, nuclear membrane fluorescence; CF, cytoplasmic fluorescence; CM, cell membrane fluorescence; LF, luminal (extracellular) fluorescence.

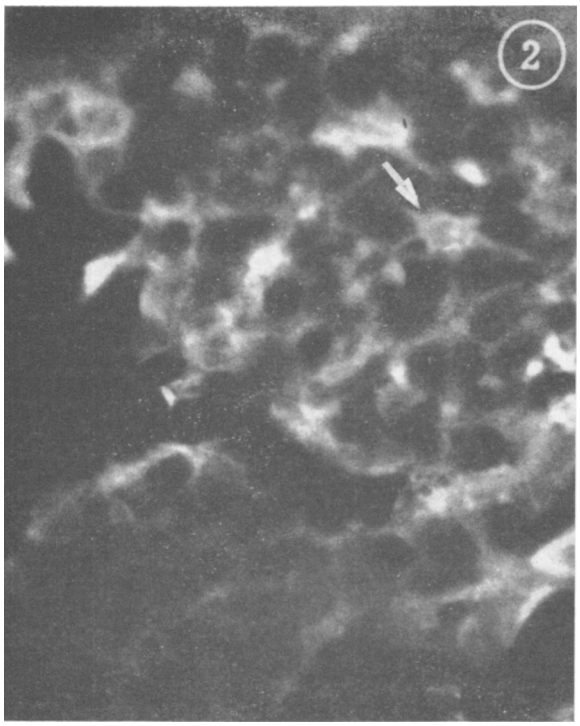

FIG. 2. Indirect immunofluorescent staining of winter tumor (VR9-70 TCI) cells: This sample displays nuclear (arrow), as well as cytoplasmic localization of antigen; approx ×350.

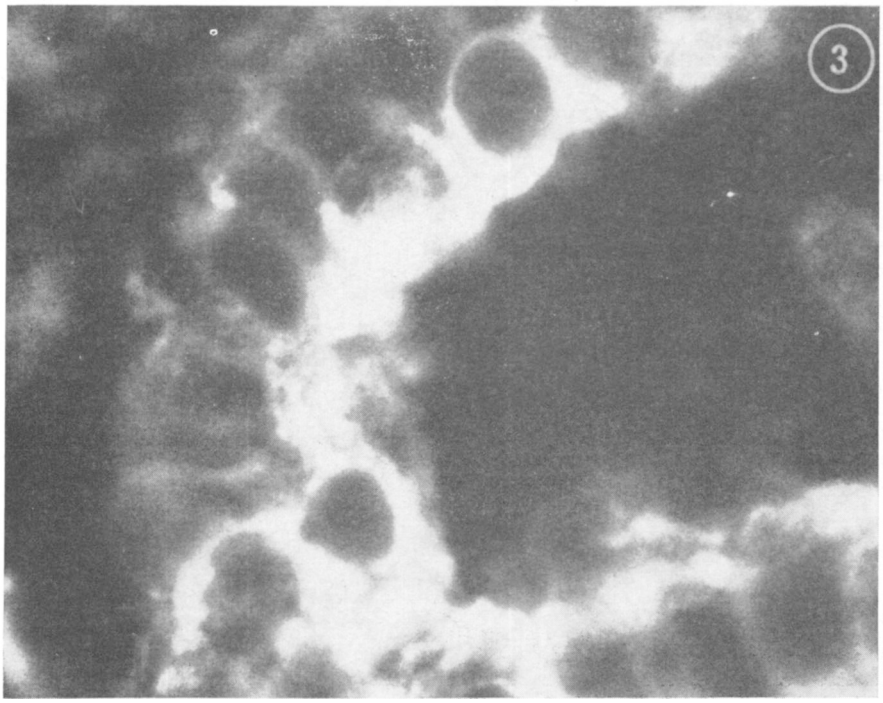

FIG. 3. Indirect immunofluorescent staining of winter tumor (VR10-69 KCI) cells: In this preparation, the fluorescence is especially prominent in the cytoplasm of cells along the luminal surface; approx ×1000.

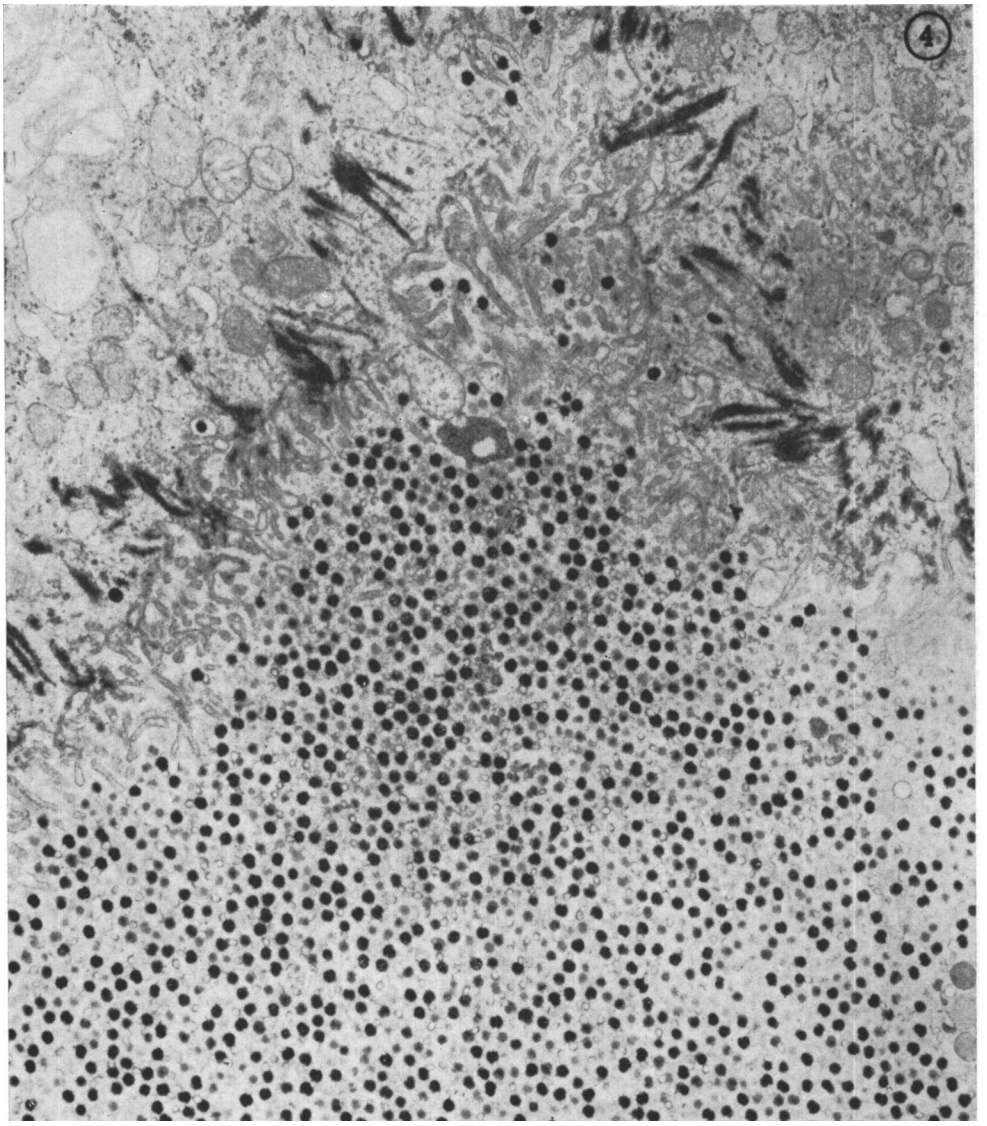

FIG. 4. Electron microscope section of winter tumor (VR9-70 UCI) illustrating herpesvirus particles filling the lumen of a tumor tubule; approx ×9450.

homogenates alone showed no activity against frog kidney antigens and slight activity against summer (nonviral) tumor antigens (arrow). This activity against summer tumor was removed by absorption with a summer tumor homogenate to yield the antiserum employed in the immunofluorescence studies.

Winter tumor frozen sections gave bright apple-green fluorescence when stained with IRS in the indirect immunofluorescence test. Intranuclear, as well as intracytoplasmic, fluorescence was detected in most winter tumor samples (Fig. 2). This may reflect developmental stages of vegetative virus production. Normal kidney and summer (nonviral) tumor sections consistently gave negative fluorescence. The proportion of cells staining and their location, with respect to tumor histology, differed with the individual tumors tested (Table I). One sample demonstrated bright cytoplasmic fluorescence in cells lining the lumen (Fig. 3). Oftentimes bright fluorescence was observed within the lumina of tumor tubules. Subsequent electron micros-

copy confirmed the concentration of virus particles in the lumina of these tumors (Fig. 4). Some tumor samples displayed bright fluorescence in a large percentage of cells throughout the section. Electron microscopy again showed this sample to have a similar pattern of virus-containing cells.

Discussion. The exact nature of the antigen being detected has not yet been elucidated. That it is an antigen of the herpesvirus which characterizes the winter state of the Lucké tumor is supported by the following evidence. First, the antiserum was prepared against purified virus fractions and, secondly, summer (nonviral) tumor antigens were absorbed out. Thirdly, the pattern of cells containing virus particles as shown by electron microscopy corresponded to the pattern of cells that were positive by immunofluorescence. Furthermore no fluorescence was observed in cells that were known to lack herpesvirus (*e.g.*, normal kidney and summer tumor). Finally similar studies using antiserum prepared against purified forms of the E-B and Marek's disease herpesviruses show fluorescing cells to contain virus (10, 11). The immunofluorescence test reported has proven to be specific for the virus-containing state of the Lucké tumor and thus far has been successfully used in quickly detecting the virus-containing nature of various tumor samples.

Summary. Antiserum prepared against purified herpesvirus fractions of the Lucké tumor has been used in detecting the virus-containing nature of various tumor samples. Bright fluorescence of winter tumor cells was observed when reacted in the indirect immunofluorescence test with the prepared antiserum. No staining was observed in tumor samples not harboring the herpesvirus or in normal frog kidney sections. The evidence that the antiserum is specific for a frog herpesvirus antigen(s) is discussed.

The authors thank Mrs. Deborah Ramsey and Mrs. Michele St. Onge for their excellent technical assistance and care of the animals. The aid of John Delfs and John Wallace in the early phases of this study is gratefully acknowledged.

1. Mizell, M., *in* "Biology of Amphibian Tumors" (M. Mizell, ed.), pp. 1–25. Springer-Verlag, New York (1969).

2. Mizell, M., Toplin, I., and Isaacs, J. J., Science **165**, 1134 (1969).

3. Epstein, M. A., Achong, B. G., Churchill, A. E., and Biggs, P. M., J. Nat. Cancer Inst. **41**, 805 (1968).

4. Burmester, B. P., Witter, R. L., Nazerian, K., and Purchase, M. G., *in* "Biology of Amphibian Tumors" (M. Mizell, ed.), p. 460. Springer-Verlag, New York (1969).

5. Richey, D. J., *in* "Biology of Amphibian Tumors" (M. Mizell, ed.), p. 469. Springer-Verlag, New York (1969).

6. Epstein, M. A., Henle, G., Achong, B. G., and Barr, Y. M., J. Exp. Med. **121**, 761 (1965).

7. Toplin, I., Brandt, P. M., and Sottong, P., *in* "Biology of Amphibian Tumors" (M. Mizell, ed.), p. 348. Springer-Verlag, New York (1969).

8. Rafferty, K. A., Cancer Res. **24**, 169 (1964).

9. Nairn, R. C., "Fluorescent Protein Tracing," p. 503. Livingstone, Edinburgh (1964).

10. Epstein, M. A., and Achong, B. G., J. Nat. Cancer Inst. **40**, 593 (1968).

11. Purchase, M. G., J. Virol. **3**, 557 (1969).

Received Oct. 19, 1971. P.S.E.B.M., 1972, Vol. 139.