The Tumor Imprint Technique For Demonstrating SV40 T Antigen By Immunofluorescence[1] (36716)

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A new intranuclear antigen known as T or "tumor," appears in various mammalian cells, when they are transformed neoplastically (1–6) *in vivo* or *in vitro* or, when they are infected productively (7, 8), by the oncogenic DNA agent simian virus 40 (SV40). Although this cellular neoantigen is virus-specific, it is not a viral capsid antigen. Its presence in neoplastic cells of various species is evidence that its synthesis is under the control of the viral genome, whose incorporation within the cell genetic apparatus (9) may be indicative of a causal relationship between the virus and the initiation and maintenance of the oncogenic state.

The complement fixation test (1–4) was first employed in detecting T antigen. However, the immunofluorescence technique (5, 6), which measures the same antigen (8, 10), is being utilized presently, primarily because it is simpler to perform. In this test, tumor cells grown in stationary cultures on cover glasses are assayed for T antigen with sera derived from adult animals bearing SV40-induced tumors. Problems arise whenever tumor cells to be tested can not grow as monolayers *in vitro,* either as a result of inadequate cultural conditions or, because they lack the capacity to attach themselves on glass or, at times, on account of tumor ulceration accompanied by bacterial putrefaction. The best way to circumvent these technical limitations is to employ histological sections of tumors, cut in the fresh frozen state in a cryostat. However, this approach is cumbersome, time consuming and may lead, in some instances, to contamination by the oncogenic virus of equipment used. In order to avoid these difficulties, we have adapted the tumor imprint technique to the indirect immunofluorescence test (11, 12). A description and evaluation of the procedure follows.

Materials and Methods. Tumors. Three-week-old male Syrian golden hamsters were inoculated intravenously, via the femoral vein, with $10^{8.5}$ median tissue culture infective dose ($TCID_{50}$) of SV40 stock virus, strain VA 45-54,[3] suspended in 1 ml of culture medium. Four to 6 months later, 85–95% of the animals developed malignant neoplasms, diagnosed microscopically as lymphocytic leukemia, lymphosarcoma, reticulum cell sarcoma, anaplastic sarcoma and osteosarcoma (13).

Imprints. Imprints or "impression smears" of the above SV40-induced neoplasms were prepared in the following manner. The primary or metastatic tumor or, an organ or a lymph node infiltrated by neoplastic cells, was held with a pair of toothed forceps and its freshly cut surface was touched gently, several times, to different areas of a number of 22 mm^2 cover glasses. An alternative and somewhat preferable method was to touch the cover glass to the cut surface of the tumor or organ, while the latter was held stationary. An attempt was made to exert as little pressure as possible, in order to avoid distortion of the cells or disruption of their spatial interrelationship.

All cover glasses with the imprinted tumor cells were allowed to air dry. They were fixed in acetone, at room temperature, for 5

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[3] SV40, strain VA 45-54, was obtained originally from Dr. M. R. Hilleman, of the Merck Institute for Therapeutic Research, West Point, PA.

min. When the acetone had evaporated, they were wrapped in lint-free paper, labeled, placed in jars containing "Drierite" (anhydrous $CaSO_4$), screwcapped tightly and stored at —70°, until such time when an immunofluorescence test could be performed.

Sera. One milliliter of blood was collected by an intracardial puncture from each one of a number of adult male Syrian golden hamsters, while they were kept under anesthesia. The sera derived from these blood samples were pooled, dispensed in 0.5 ml aliquots and stored at —20°. This was known as the "pretumor" serum. The anesthetized animals were implanted intramuscularly with 1–2 × 10^6 SV40-transformed homologous embryonic skin and subcutaneous tissue cells, line B (14). These cells were shown to contain SV40 T antigen, but were found to lack the capacity to produce infectious SV40 when grown in culture, in the absence of indicator grivet monkey kidney cells. Two to 3 months later, hamsters bearing large tumors were anesthetized and exsanguinated. Sera derived from these blood samples were pooled and stored at —20° in aliquots of 1 ml. This was known as the "posttumor" serum. Neither the "pre-" nor the "posttumor" serum, when diluted 1:4, neutralized 100 $TCID_{50}$ of SV40 stock virus. The fluorescein-labeled anti-hamster globulin goat serum (conjugate), obtained commercially,[4] was stored undiluted, at —20° in aliquots of 0.5 ml.

Indirect immunofluorescence technique. Cell imprints held at —70° were allowed to reach room temperature while still inside the screwcapped jars. Each cover glass with imprinted cells was overlayed with 3–4 drops of "posttumor" serum. The latter was used either undiluted or, when of high anti-T titer, it was diluted 1:2 or 1:4 with phosphate buffered saline (PBS, pH 7.2–7.3). In each test, duplicate imprint preparations of some of the tumors were treated with undiluted "pretumor" serum. In addition, known SV40-T-positive (line 2K) and SV40-T-negative (line I) hamster embryo cells (15), grown on cover glasses in culture, were rinsed twice in PBS prior to acetone fixation for 5 min and treated with "pre-" or "posttumor" serum, respectively. These preparations were utilized as the SV40 T "positive" and "negative" controls.

The cover glasses bearing the various cells and their overlayed sera, while supported horizontally on a rack, were placed in a humidified chamber and incubated at 37° for 1 hr. Then, they were rinsed for 5 min, twice, in PBS and were overlayed for a second time, but with 3–4 drops of fluorescein-labeled anti-hamster goat globulin freshly diluted 1:4 or 1:8 with PBS. They were incubated in the same humidified chamber at 37° for 1 hr, rinsed twice in PBS as before and mounted while still wet on microslides in buffered glycerin (9:1, glycerin to PBS), pH 8.2–8.5 (2 drops of 0.1 *N* NaOH to 10 ml of buffered glycerin).

The edges of each cover glass were sealed to the microslide with a paraffin–Vaseline (3:1) mixture, while it was kept melted at approximately 60°. The exposed surface of each cover glass was wiped clean of dried salts with a cotton swab, moistened in distilled water. The preparations were ready at this point to be examined under the fluorescence microscope, employing darkfield condenser. If the microscopical examination had to be postponed, the slides were stored in the refrigerator at 4°, with no appreciable loss of fluorescence activity, over prolonged periods.

Results and Discussion. Multiple imprints of over 200 primary hamster neoplasms (Fig. 1–4) induced by SV40 *in vivo* and of approximately 100 of their transplants, as well as of various organs and lymph nodes (Fig. 5), that were involved by the neoplastic process, were examined for SV40 T antigen by immunofluorescence. On the basis of experience derived from evaluating this material, it can be stated with assurance that the tumor imprint technique offers the following major advantages over that which utilizes cells grown in culture.

First, the cytoplasmic and nuclear features of the imprinted tumor cells remain intact. This is not the case with neoplastic cells grown in culture, since their morphological characteristics are usually modified to the extent that they appear either fibroblastoid

[4] The conjugate was purchased from Antibodies Incorporated, Davis, CA 96616.

FIGS. 1–5 show imprints of neoplasms induced in hamsters by the oncogenic DNA virus SV40.

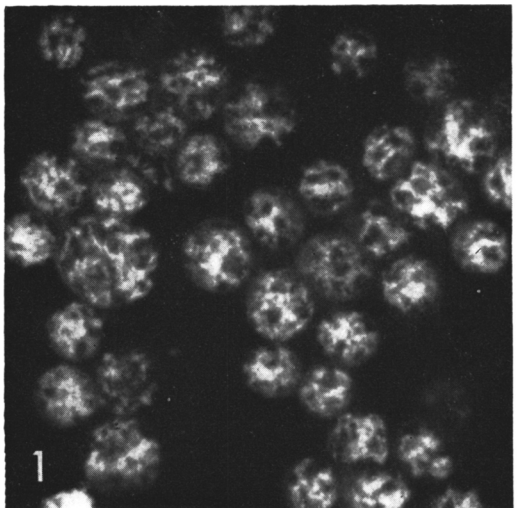

FIG. 1. SV40-lymphocytic leukemia.

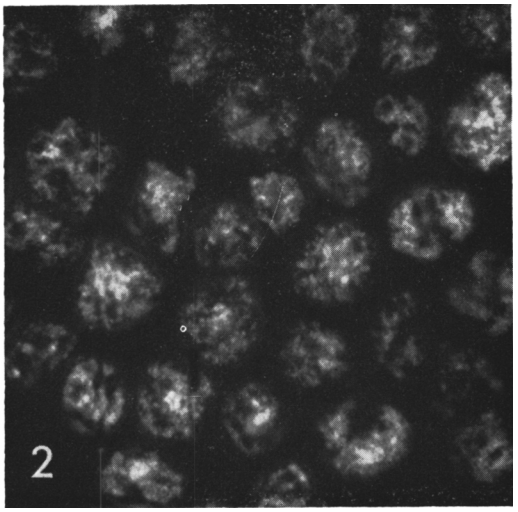

FIG. 2. SV40-lymphosarcoma.

or epithelioid, features that may be unrelated to the cell phenotype.

Second, the *in vivo* spatial relationship of tumor cells to one another and to the normal fibroblastic or phagocytic stromal cells, is well preserved in imprint preparations while it is completely destroyed following their cultivation *in vitro*. Preservation of cellular interrelationships is of paramount importance if one wishes to study the mode of origin of a neoplasm, as it is revealed by its *in situ* stage of growth (Fig. 5).

Third, examination of leukemic or lymphosarcomatous cells for virus-mediated neoantigens by immunofluorescence has been always a difficult if not an impossible task, since such cells can not grow as monolayers in culture. One, then, has to resort to examining histological sections of these neoplasms, cut in the fresh frozen state in a cryostat, a procedure that may be cumbersome, as well as hazardous to the worker performing it,

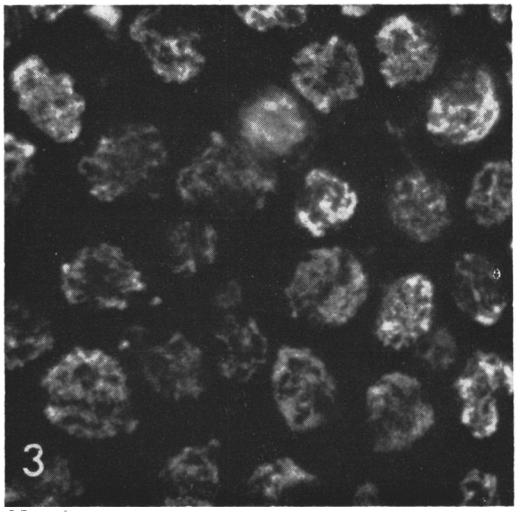

FIG. 3. SV40-reticulum cell sarcoma.

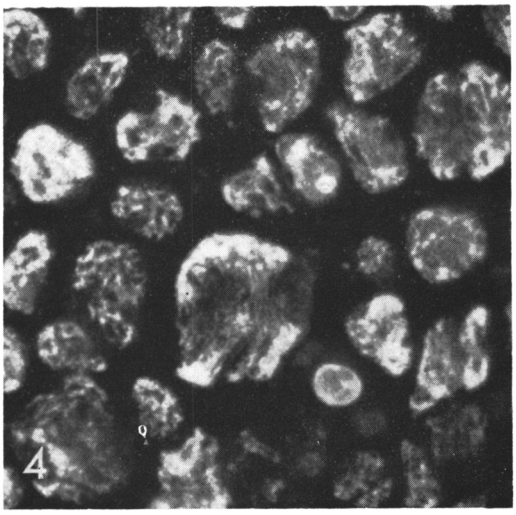

FIG. 4. SV40-anaplastic sarcoma.

since some of the neoplastic cells may be spontaneously virogenic. However, the tumor imprint technique makes examination of such cells an easy task (Figs. 1, 2), while at the same time long delays from growing cells *in vitro* and expenses encountered from such efforts are eliminated.

Fourth, tumor ulceration with superimposed bacterial contamination may prevent the growth of neoplastic cells in culture, since the microorganisms present could interfere with cell survival and proliferation. Bacterial putrefaction of tumor material has no deleterious effect on imprint preparations, since the cells to be examined are transferred directly on cover glasses without prior *in vitro* cultivation.

Finally, when one compares the tumor imprint technique (Fig. 1–5) with that of cells grown in culture (Fig. 6), in regards to brightness and distinctness of SV40 T antigen achieved, T-positive nuclei of *in vitro* grown cells which are more flattened out, may appear somewhat brighter and sharper under fluorescent light than positive nuclei of imprinted cells that have a more pronounced three dimensional outline. These quantitative differences, however, are not of the magnitude that could influence the outcome of the microscopical evaluation to any appreciable degree.

Summary. A quick, reliable and inexpensive test is described for identifying the T or "tumor" antigen, in cells rendered neoplastic by the oncogenic DNA virus SV40. The approach is based on the feasibility of adapting the tumor imprint technique to the indirect immunofluorescence test. This method is of particular advantage when one wishes to examine leukemic or lymphosarcomatous cells, which lack the capacity to grow as monolayers in culture.

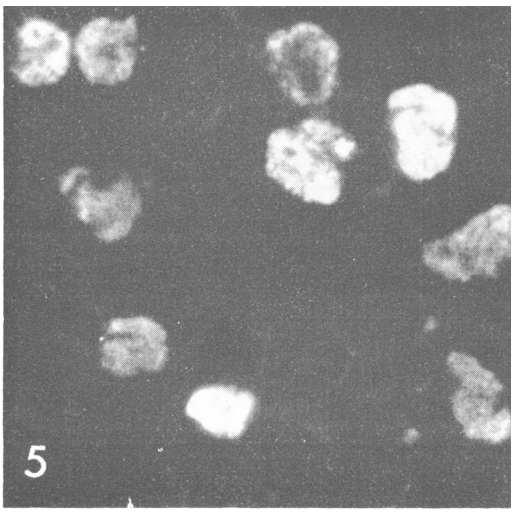

FIG. 5. SV40-reticulum cell sarcoma *in situ*.

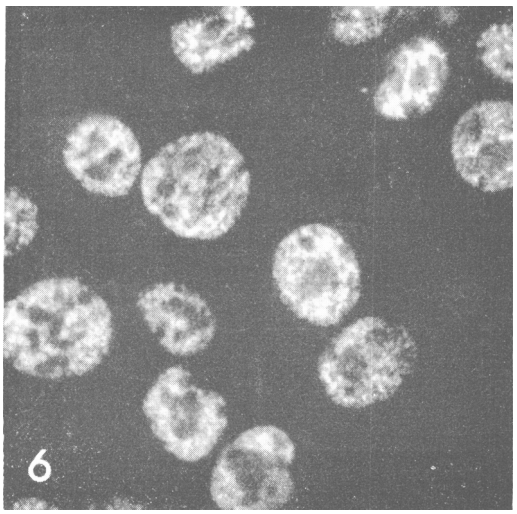

FIG. 6. SV40-transformed hamster embryonic skin and subcutaneous tissue cells grown *in vitro*. The white (reacting) nuclei of the neoplastic cells seen in all six figures, have been stained by indirect immunofluorescence for SV40 T or "tumor" antigen. Normal lymphocytes with nonreacting nuclei are seen among the neoplastic cells in the lymph node imprint, Fig. 5. ×800.

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