Biophys. Acta, in press.
4. ———, ibid., 1965, v103, 325.
5. Pieber-Perretta, M., Rudolph, W., Perretta, M., Hodgson, G., ibid., Acta, 1965, v95, 360.
6. Lowy, P., Borsook, H., Erythropoiesis, Jacobson & Doyle, ed., Grune & Stratton, N. Y., 1962, p33.
7. Fuyioka, M., Koga, M., Lieberman, E., J. Biol. Chem., 1963, v240, 3401.
8. Bosco, R., Verly, W. G., Maisin, R., Clin. Chim. Acta, 1963, v8, 58.
9. Rambach, W. A., Shaw, R. A., Cooper, J. A., Alt, H., Proc. Soc. Exp. Biol. & Med., 1958, v99, 482.
10. Hayhoe, F. G., Quaglino, D., Nature, 1965, v205, 151.
11. Harris, H., Progress, Nucleic Acid Res., Davidson & Cohn, ed., Academic Press, 1963, v2, 19.
12. Kurtides, E. F., Rambach, W. S., Alt, H., Wurster, J. C., Lab. Clin. Med., 1963, v61, 23.
13. Alpen, E. L., Cranmore, D., Ann. N. Y. Acad. Sci., 1959, v77, 753.

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Polyoma Virus-Induced "Complement-Fixing Antigen" in Tumors and Infected Cells as Detected by Immunofluorescence.* (31920)

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The cellular response to infection with DNA oncogenic viruses may be expressed in two different ways. In one case the cells lyse as a result of virus multiplication while in the other the intervention of the viral genome brings about changes leading to malignant transformation of the cells. There is as yet no evidence available on the persistence of the complete viral genome in the transformed cells; however, the virus leaves traits in the host cells in the form of specific antigenic substances by which the cell-virus interaction can be identified. One of these antigens, "tumor" antigen(1), detectable by the complement-fixation test, will be referred to as induced complement-fixing antigen (ICFA)(2). The presence of ICFA has been demonstrated during lytic interactions in cells infected by SV40 and adenoviruses, as well as in the tumors that these viruses induce and that are free of infectious activity(1-8). As shown by immunofluorescent technique ICFA has nuclear localization, and, at least in the case of SV40, there is evidence that the antigens demonstrated by the complement-fixation and immunofluorescence tests are identical(2). Polyoma ICFA has been demonstrated by the complement-fixation test in tumors produced by polyoma(9-11) as well as in primary infected cultures(10,11), but until very recently(12-13), only in the latter case could the antigen be shown by immunofluorescence.

From the results reported here it is evident that the polyoma virus (PV) ICFA can be demonstrated at a cellular level in polyoma-induced rat, mouse, and hamster tumors and that in embryonic tissue cultures of the same species infected with PV, the proportion of ICFA-containing cells greatly exceeds that of cells positive for viral antigens.

Materials and methods. Viruses. Two different pools of polyoma virus were used: One (P-178) was derived from a wild strain ob-

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tained from Dr. E. A. Mirand, Roswell Park Institute, Buffalo, N. Y., and had a titer of 10^8 plaque-forming units (PFU) per ml; the other (P-189) was derived from a small plaque variant received from Dr. R. Dulbecco, Salk Institute, La Jolla, Calif., and had a titer of $10^{7.7}$ PFU per ml.

Tumors. Rat kidney fibrosarcomas were induced in newborn Lewis inbred rats by intraperitoneal inoculation with 0.1 ml of P-178 virus. Hamster subcutaneous fibrosarcomas were induced by subcutaneous inoculation of newborn Lakeview hamsters with 0.1 ml of P-189 virus. The polyoma-transformed hamster line (P-147) was tested at the 25th passage *in vitro*(14). The mouse tumor was a transplantable polyoma tumor (#695) obtained from Dr. K. Habel, Inst. of Allergy and Infectious Diseases, U.S. P.H.S., Bethesda, Md., and transplanted in isologous C_{57}/Bl mice in our laboratory for a period of over 4 years(15).

Tissue culture. Primary monolayer cultures were prepared from inbred rat (strain DA/Ss) embryos at about 17 days of gestation, from Lakeview hamster embryos at about 12 days of gestation and from mouse (strain C_3H) embryos at about 16 days of gestation. The cells were trypsinized and grown on cover glasses in Falcon plastic tissue culture dishes, seeding 4×10^6 cells per dish in 5 ml of medium.

Rat, hamster, and mouse tumors of various sizes (0.5-3 cm) were aseptically removed and trypsinized in a 0.25% trypsin and 0.1% collagenase (Nutritional Biochemicals) phosphate buffered salt solution for 90 minutes on a magnetic stirrer at 37°C(16). The tumor cells and hamster line (P-147) were grown under the same conditions as the embryo cultures being subcultured every 4-6 days. Eagle's medium containing a 2-fold concentration of vitamins and amino acids plus 10% horse serum was used for all the embryo and hamster tumor cultures. For the rat and mouse tumors and the hamster line, 10% calf serum was used instead. The media were supplemented with antibiotics (100 μg/ml penicillin and 100 units/ml streptomycin). The cultures were kept in a humidified incubator at 37°C with a flow of 10% CO_2 in the air.

Anti-PV ICFA serum. The antiserum was produced by repeated immunization of weanling hamsters(17) over an extended period of time with a transplantable polyoma hamster tumor homogenate emulsified at 1:1 proportion with Freund's complete adjuvant. Approximately 1 ml of the mixture was inoculated intraperitoneally or in the footpads. The CF titer of the injected material ranged from 1:8 to 1:32. At least 6 injections were required to produce antibody of the desired high titer in a large proportion of the animals, although an increasing proportion of sera developed anticomplementary activity during immunization. The serum was stored at —70°C. No antiviral antibody was detected in a random sample of sera prepared in this fashion.

Complement-fixation test. The micro titer complement-fixation test was done according to the technique of Sever(18), using a 20% tumor homogenate.

Immunofluorescent staining. The PV-ICFA was demonstrated by the indirect method (19). The coverslip cultures were washed twice in phosphate buffer saline, dried in air at room temperature and fixed in acetone-methanol mixture (7:3) at —20°C for 10 minutes. They were then incubated successively with anti-ICFA hamster serum diluted 1:5 and antihamster gamma globulin labelled with fluorescein isothiocyanate (courtesy of Dr. R. E. Wilsnack, Baltimore Biological Laboratory, Baltimore, Md.) at 37°C for 30 minutes. After each time they were washed in phosphate buffered saline for 10 minutes and dried in air. As mounting media, either Elvanol or 25% glycerol in phosphate buffered saline was used.

The PV antigen (PV-A) was stained with rabbit fluorescein-labelled anti-PV serum(20), prepared by immunization with partially purified virus. The serum did not have any anti-PV-ICFA activity.

Results. Specificity of immunofluorescent staining. The serum used for immunofluorescent studies fixed complement in the presence of polyoma tumor homogenates at a dilution of 1:160 while no reaction occurred with homogenates of tumors induced by SV40,

TABLE I. Complement-Fixing Activity of Anti-PV-ICFA Serum with Tumors Induced by Different Viruses.

Antigens*	Serum dilution 20	40	80	160	320
Polyoma	4	4	4	4	1
SV_{40}	0 →				
RSV (Schmidt-Ruppin)	0 →				
Adeno 7	0 →				
Adeno 12	0 →				

* 20% tumor homogenates used at 4 units based on homologous titrations.

Rous sarcoma virus (Schmidt-Ruppin), adenovirus 7 and adenovirus 12 (Table I).

This serum did not react in the immunofluorescent test with several tissue culture preparations of hamster SV40 tumors, noninfected mouse, hamster, and rat embryo cells and L-cells. No immunofluorescence was observed in polyoma-infected or polyoma tumor cells treated with normal or anti-SV40-ICFA hamster serum. Absorption of the antiserum with virus free polyoma-induced tumor removed the complement-fixing and immunofluorescence activity.

Rat primary tumors. Four rat kidney primary tumors that had been *in vitro* between 10 days and 1 month showed a variable proportion of PV-ICFA positive cells with values ranging from 50% to 95%. The antigen had a typical nuclear localization appearing as granules distributed throughout the entire nucleus except the nucleoli (Fig. 1). The concentration of the fluorescent granules varied in different cells. In a proportion of the cells they were dense enough to form a diffused type of fluorescence while in a minority of cells they were barely detectable. No viral antigen was detected by immunofluorescence in any of these cultures.

Mouse transplantable tumor. This tumor was tested 4 days after explantation and 100% of the cells contained PV-ICFA antigen. The fluorescence was granular with similar high intensity in all the cells. These tumor cells were negative for viral antigen.

Hamster primary tumors. Four primary tumors were tested for the PV-ICFA at 2, 6 and 10 days after explantation. In 3 tumors the percentage of positive cells varied at different days, ranging from 30% to 80%. In the fourth tumor all the cells were invariably positive. On the whole, fewer fluorescent granules were observed in the hamster than in the rat and mouse tumors. Occasionally the immunofluorescence was represented by granules dispersed (Fig. 2) throughout the nucleus. As estimated from the size and density of the fluorescent granules, the greatest amounts of ICFA were constantly present in the hamster tumor in which all cells were positive.

Polyoma-transformed hamster line (P-147). The cells of this line had a type of fluorescence similar to that observed in other hamster tumors. Although all the cells were positive for ICFA, the concentration of granules per nucleus was low and in some cells barely detectable.

Cultures infected by polyoma virus. Mouse embryo cultures. Confluent monolayers of primary mouse embryo cells were infected with polyoma virus (P-189) at a multiplicity of 20 PFU per cell and tested for the PV-antigen and ICFA at different intervals after infection. The results are summarized in Table II.

The appearance of ICFA preceded that of the viral antigen by at least 6 hours. At all times the number of ICFA-containing cells exceeded considerably the number of PV-antigen-positive cells. At 2 and 3 days after infection the proportion of cells positive for the PV-antigen and ICFA increased abruptly, suggesting that more than a single proliferation cycle of the virus may have occurred.

TABLE II. Percentage of ICFA and Viral Antigen Containing Cells in PV-Infected Mouse, Rat, and Hamster Embryo Cultures.

Species	Antigens	Hours after infection 18	24	44	72	96
Mouse	ICFA	.92*	3.88	11.4	25.2	N.T.
	Viral	0	.46	3.86	7.84	N.T.
Hamster	ICFA	N.T.†	3.24	11.2	8.6	8.28
	Viral	N.T.	0	.28	.58	.18
Rat	ICFA	N.T.	.24	4.36	8.24	10.5
	Viral	N.T.	0	.10	.48	.36

* Confluent monolayers were infected with polyoma virus (P-189) at a ratio of 20 pfu per cell. The percentage of positive cells was obtained by scoring 50 microscopic fields in 2 coverslips, each field containing an average of 100 cells.

† N.T. = not tested.

The ICFA in certain cells was in the form of little spots found only in a part of the nucleus. More often, however, it was diffused throughout the whole nucleus (Fig. 3).

Rat and hamster embryo culture. In further experiments, rat and hamster primary con-

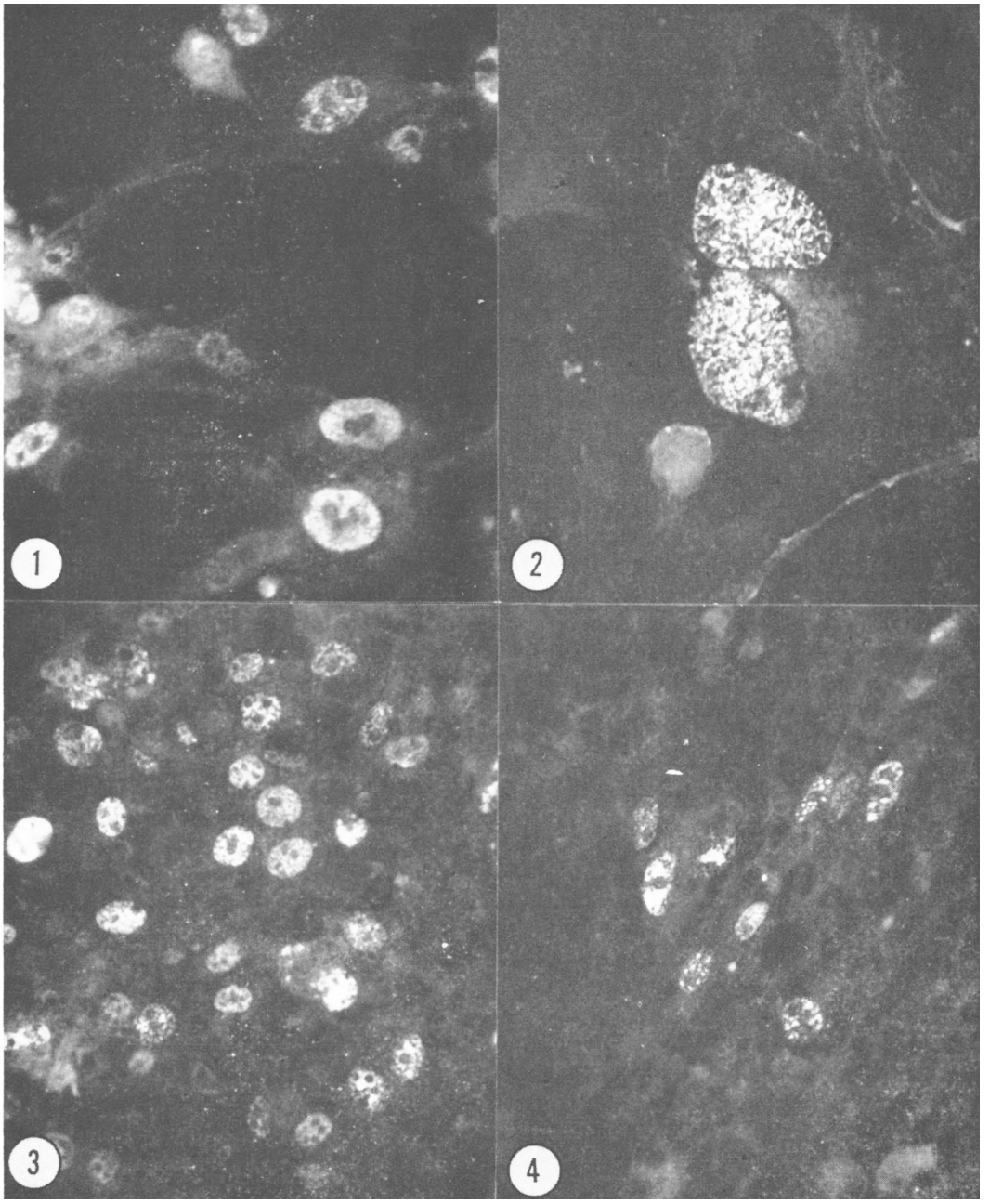

FIG. 1. Primary rat polyoma tumor. Note diffused and granule type of fluorescence.
FIG. 2. Primary hamster polyoma tumor. Both nuclei of a giant cell show numerous fluorescent granules.
FIG. 3. Mouse primary culture 2 days after infection.
FIG. 4. Rat primary culture 3 days after infection. Note variable concentration of antigen in different nuclei.

fluent monolayers were infected with P-189 polyoma virus at a multiplicity of 20 PFU and samples were examined at various intervals for viral antigen and ICFA. As shown in Table II, the PV-ICFA appeared prior to the viral antigen in rat and hamster cells similarly as in the mouse cells. The viral antigen was synthesized in a much lower proportion of rat (Fig. 4) and hamster cells than in mouse cells, thus indicating that in cells of these species a productive interaction with polyoma virus may occur, although to a very low extent. In rat and hamster cultures the proportion of PV-ICFA positive cells continued to increase or stayed at a high level up to 96 hours postinfection while the proportion of cells containing viral antigen remained constantly low.

Discussion. These results indicate that PV-ICFA can be demonstrated by immunofluorescence in PV-primary rat and hamster tumors as well as in a transplantable mouse tumor maintained for several years. The PV-ICFA has a typical nuclear localization appearing in either a diffuse or granular form; nucleoli and cytoplasm are consistently negative. Among the various tumors, the concentration of fluorescence was lower in hamster than in rat and mouse tumors. Variation in intensity and distribution of immunofluorescence was observed within cells of the same preparation, the greatest variability being found in the primary rat tumors. The finding that in most of the primary tumors not all the cells gave a positive reaction for ICFA might be ascribed either to the presence of non-tumor cells that could have been present in the freshly explanted tumor cultures, or to the fact that in certain cells the concentration of the antigen remained below the threshold sensitivity of the test.

Takemoto *et al*(13) have recently reported that a heat labile factor present in fresh hamster serum is required for the optimal immunofluorescent staining of ICFA in polyoma-induced tumor and that the type of immunofluorescence (diffuse or granular) depends on the presence of this factor. It was also observed in our laboratory that immunofluorescence reactivity could be restored by addition of fresh hamster serum to an anti-SV40-ICFA serum that had lost activity upon prolonged storage (Porter, D., unpublished observation). From our findings it appears, however, that both types of fluorescence may be shown in polyoma tumor cells with a high titer anti-PV-ICFA serum, and that granular *versus* diffuse fluorescence may reflect actual differences in antigen concentration.

The previous failure to demonstrate PV-ICFA by immunofluorescence in virus-free tumors(11-12) could be accounted for by the fact that the available antisera were of low titer.

The PV-ICFA was also demonstrated by immunofluorescence in primary PV-infected mouse, rat, and hamster embryo cells. In all of these cultures synthesis of this antigen preceded that of viral antigen. Similar results have been obtained with various tissue cultures infected with SV40 and adenoviruses (2,6,21,22). Furthermore, in the polyoma infected cultures the percentage of ICFA positive cells at any one time postinfection greatly exceeded that of viral antigen positive cells. This was particularly true in hamster and rat cells, in which the ratio of PV-ICFA to PV-A positive cells, at the multiplicity of infection used, was about 40. The presence of PV-ICFA can then be taken as a better measure of the number of cells infectable in a population. It has been previously shown(14,23, 24) that hamster and rat embryo cells infected at a low multiplicity (5 PFU) transformed without detectable virus growth; however, as shown in these experiments and those reported by Sheinin(25), when rat cells were infected at a high multiplicity, a minor fraction (0.1-0.5%) of the cell population supported viral growth. It is also possible that cells from different rat strains differ in their sensitivity to infection.

PV-ICFA appears to be a stable component persisting for years in the transplantable polyoma mouse tumor and in polyoma transformed virus-free hamster and mouse lines (10,11), thus PV-ICFA containing cells must be able to multiply.

It has been recently demonstrated that in human cultures infected with SV40 a proportion of cells which do synthesize SV40-ICFA but not viral antigen, also synthesize DNA

and divide(26). The hypothesis was formulated that the transforming cells originate from the pool of cells in which transcription of the viral genome occurred but was not adequate for complete viral synthesis. The findings that also in polyoma-infected rat and hamster cultures, which are transformable by the virus, a considerable proportion of the cells is PV-ICFA positive but viral antigen negative, tend to support this hypothesis. Thus, the appearance of PV-ICFA, not followed by viral antigen synthesis, may be regarded as an early marker for events of the cell-virus interaction which may lead to transformation.

Summary. Polyoma virus-induced complement-fixing antigen (PV-ICFA) has been demonstrated by immunofluorescence in polyoma primary rat kidney and hamster subcutaneous fibrosarcomas, in one polyoma transplantable mouse tumor and in one polyoma-transformed *in vitro* hamster cell line. The antigen has a typical nuclear localization appearing either in a granular or in a diffused form throughout the whole nucleus except for the nucleoli. A lower concentration of ICFA was found in the hamster tumors than in the rat and mouse tumors. The PV-ICFA was also demonstrated in polyoma-infected mouse, hamster, and rat embryonic tissue cultures. In primary infected cultures of all three species, the synthesis of PV-ICFA preceded that of viral antigen (PV-A). Many more cells synthesized PV-ICFA than PV-A. The difference was especially high in the hamster and the rat cultures in which the ratio of PV-ICFA to PV-A positive cells averaged about forty.

1. Huebner, R. J., Rowe, W. P., Turner, H. C., Lane, W. T. Proc. Nat. Acad. Sci., 1963, v50, 379.

2. Gilden, R. V., Carp, R. I., Taguchi, F., Defendi, V., ibid., 1965, v53, 684.

3. Black, P. H., Rowe, W. P., Turner, H. C., Huebner, R. J., ibid., 1963, v50, 1148.

4. Pope, J. H., Rowe, W. P., J. Exp. Med., 1964, v120, 121.

5. Sabin, A. B., Koch, M. A., Proc. Nat. Acad. Sci., 1964, v52, 1131.

6. Rapp, F., Kitahara, T., Butel, J. S., Melnick, J. L., ibid., 1964, v52, 1138.

7. Pope, J. H., Rowe, W. P., J. Exp. Med., 1964, v120, 577.

8. Rapp, F., Butel, J. S., Melnick, J. L., Proc. Soc. Exp. Biol. Med., 1964, v116, 1131.

9. Defendi, V., Ephrussi, B., Koprowski, H., Nature, 1964, v203, 495.

10. Habel, K., Virology, 1965, v25, 55.

11. Defendi, V., Taguchi, F., Ann. Med. Exp. Fenn., 1966, v44, 232.

12. Takemoto, K. K., Malmgren, R. A., Habel., K., Virology, 1966, v28, 485.

13. ———, Science, 1966, v153, 1122.

14. Defendi, V., Lehman, J. M., J. Cell. Comp. Physiol., 1965, v66, 351.

15. Defendi, V., Room, R. A., Cancer Res., 1965, v25, 300.

16. Defendi, V., Lehman, J. M., Int. J. Cancer., 1966, v1, 525.

17. Gilden, R. V., Beddow, T., Huebner, R. J., in preparation.

18. Sever, J. L., J. Immunol., 1962, v88, 320.

19. Weller, T. H., Coons, A. H., Proc. Soc. Exp. Biol. Med., 1954, v86, 789.

20. Sachs, L., Fogel, M., Virology, 1960, v11, 722.

21. Bissett, M. L., Payne, F. E., J. Bact., 1966, v91, 743.

22. Riggs, J. L., Takemori, N., Lennette, E. H., Proc. Soc. Exp. Biol. Med., 1965, v120, 832.

23. Sachs, L., Medina, D., Nature, 1961 v189, 457.

24. Medina, D., Sachs, L., Brit, J. Cancer, 1961, v15, 885.

25. Sheinin, R., Virology, 1966, v29, 167.

26. Sauer, G., Defendi, V., Proc. Nat. Acad. Sci., 1966, v56, 452.

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