THE JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY
Vol. 13, No. 8

Printed in U.S.A.

FLUORESCENT ANTIBODY STUDIES OF VIRUS-INDUCED TUMORS

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For some years the immunofluorescent technique, using classical antiviral immune sera, has been a useful tool in the study of tumorigenic viruses. Probably the most important example of its ability to localize antigen-antibody reaction at the cellular level was the study of Noyes and Mellors (1957) who showed that the viral antigen of rabbit papilloma virus was not present in the proliferating layer of a papilloma, but only in more superficial cells about to become keratinized. Immunofluorescent detection of polyoma virus provided the basis for a valuable study of the distribution of the virus during the acute stage of infection (Levinthal *et al.*, 1962), and the finding that Rous virus antigen is localized to the cell membrane provided the basis for a theory of its mechanism of oncogenesis (Vogt and Rubin, 1962). In quantitative studies of cell transformation by polyoma and SV40 viruses, immunofluorescent studies have provided additional quantitative parameters, such as the proportion of cells taking in virus and the proportion replicating the virus (Fraser and Gharpure, 1962).

However, in a most important area of viral oncology, classical immunofluorescence has been disappointingly negative, that is, in relation to the question of persistence of virus or viral material in virus-induced tumors. Many tumors and transformed tissue culture cell lines induced by polyoma, rabbit papilloma and SV40 viruses are devoid of infectious virus, and adenovirus-induced tumors are uniformly virus-free, but evidence from transplant rejection (Habel, 1961; Sjogren *et al.*, 1961) and special virus retrieval studies (Gerber and Kirschstein, 1962; Sabin and Koch, 1963; Black *et al.*, 1963) has given indications that viral genetic material indeed is present in the noninfectious tumors. In these systems searches for virus influence by immunofluorescent tests with antiviral immune sera have only confirmed the negative virus isolation tests. SV40 Hamster tumor cell lines in which at least the majority of cells have the unexpressed potential for producing infectious virus give no staining with antiviral serum (Pope and Rowe, 1964a); even transformed cell lines which do chronically produce virus show staining of only one cell per 10,000.

The resolution of this problem has come about as an outgrowth of the discovery by Huebner *et al.* (1963) of a previously unknown class of virus-induced antigens produced by noninfectious tumors. The basic observation is that rodents, particularly hamsters, carrying DNA virus-induced tumors develop complement fixing (CF) antibodies reactive with extracts of the tumors and also reactive with certain antigens produced by the virus during ordinary cytolytic infection. The essential point is that these antibodies are not directed against the classical antigens of the virus particle (with one important exception (Huebner *et al.*, 1964b)) but against virus-induced proteins which, as far as is known, are not utilized as structural components of the virus particle. These antigens, now termed neoantigens, or T antigens, are smaller than the virus, are generally more heat labile and appear to be very poorly antigenic. It is probably because of their low antigenicity that tumor-bearing animals provide the only present source of antibodies against the neoantigens, the antigen-rich tumor providing a continual source of massive antigenic stimulation.

The use of these neoantigen-reactive tumored hamster sera for immunofluorescent studies has in large part confirmed the patterns delineated by CF testing and has contributed much new information by permitting examination at the cellular level. The particular usefulness of the FA procedure has been in providing information on the proportion of cells producing the neoantigens, on the intracellular localization and morphology of the various antigens and on the quantitative aspects of their formation in infected cells.

For FA study, cells are grown on coverslips in petri dishes and fixed in cold acetone. Tumored hamster sera, selected on the basis of having high titer CF antibody against tumor extracts, are tested by the indirect FA test using fluorescein isothiocyanate-conjugated goat anti-hamster

globulin (prepared by Baltimore Biologics Laboratory). Anti-viral immune sera, reactive with the infectious virus and the classical soluble antigens but not with neoantigens, are used as direct conjugates. In much of our work rhodamine counterstaining has been used with highly satisfactory results.

FA staining of SV40-induced tumor cells has given very clearcut results (Pope and Rowe, 1964a; Rapp *et al.*, 1964a). All tumor cells show brilliant, specific staining with SV40-tumored hamster serum. The staining is restricted to the nucleus appearing as a homogeneous or slightly granular material uniformly filling the nucleus but sparing the nucleoli. In the multinucleated giant cells, which are highly characteristic of SV40 tumors, every nucleus is intensely stained. Most important, identical results are obtained when the SV40-tumored hamster serum is used to stain any species cell transformed by SV40; hamster, rabbit, mouse, pig and human transformed cells all give 99 to 100% bright nuclear staining (Fig. 1). This observation has been particularly important for indicating that the neoantigen is under viral genetic control, and that presence of the neoantigen does indicate persistence of viral genes in the cells rather than a stable derepression of host genes. Further evidence for viral genetic control was provided by the demonstration that the FA-stainable antigen appeared during the acute replicative infection of SV40 in monkey and other species cells. The antigen appears in the nucleus well before viral antigen, and in its appearance and serologic reactivity is identical to that in the tumor cells.

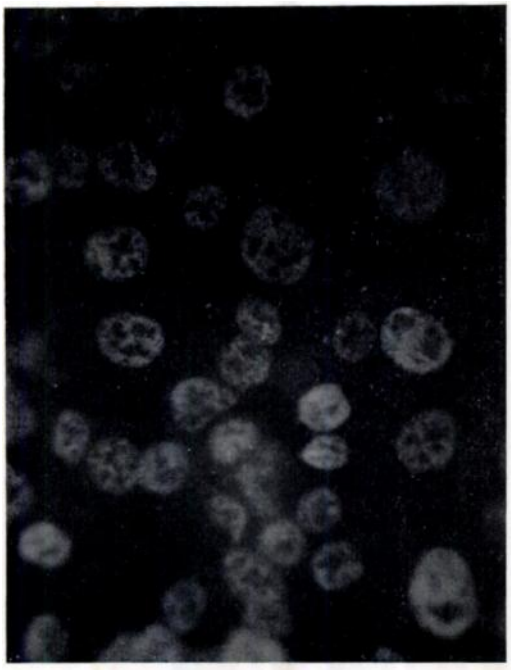

FIG. 1. SV40 Transformed human cells stained with SV40 tumored hamster serum.

In principle, the findings with adenovirus tumors (Pope and Rowe, 1964b) have been the same as with SV40, but the details are far more complex due to the great number of antigens (possibly 10 or 20) produced by the larger adenovirus genome. Tumors produced by Ad. 12, when reacted with Ad. 12 tumored hamster serum, again show specific staining in essentially 100% of cells, regardless of species of origin (hamster, rat or mouse). The morphology and distribution of the antigen is quite different from that of SV40; it is characteristicelly seen as numerous elongated rods, needles or fibers throughout the nucleus and cytoplasm sometimes against a homogeneously stained background. This fleck-type morphology is highly unique; it is not seen with FA staining of the classical adenovirus antigens, that is, those that make up the infectious particle, and has not been observed with any other group of viruses.

Acutely infected cells also synthesize the fleck antigens. Again, the neoantigens are formed earlier than the structural antigens, the former appearing at 6–8 hr, and the latter at 14–18 hr. The neoantigens in the nucleus show a characteristic morphologic sequence in the acutely infected cells. The antigen first appears as a few bright dots, within a few hours develops into large masses of flecks and fibrils (Fig. 2) and after another 6-12 hours or so decreases in amount and coalesces into coarse bundles and rings (Fig. 3). This is in marked contrast to the tumor cells where the antigen continually remains in the fleck form.

In monkey and hamster cells, which undergo abortive infection, that is, infection which may be accompanied by viral cytopathogenicity but with little or no production of infectious virus, the fleck-shaped neoantigens are formed as efficiently as in fully susceptible cells; but FA-stainable viral antigen does not appear (Pope and Rowe, 1964b). With both adenovirus and SV40, a comparable dissociation is produced by treating sus-

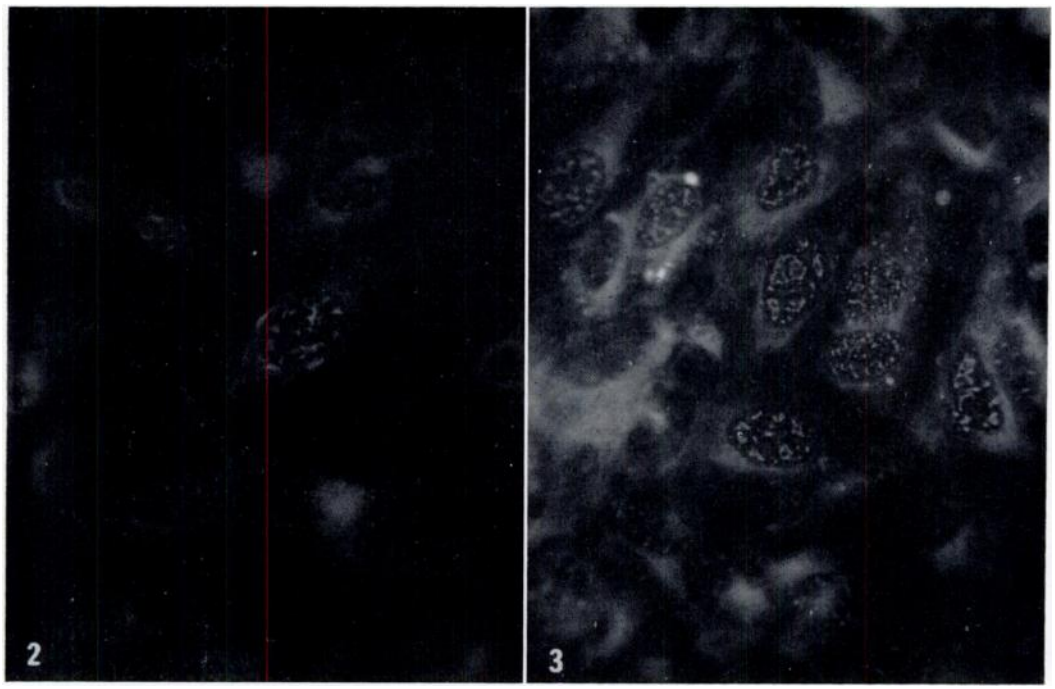

FIG. 2. Human embryonic kidney cells in tissue culture infected with adenovirus (type 12) 18 hr previously. Stained with Ad. 12 tumored hamster serum. The cell in the center shows nuclear and cytoplasmic antigen in the form of flecks and needles.

FIG. 3. Human embryonic kidney cells in tissue culture infected with adenovirus (type 7) 24 hr previously. Stained with Ad. 12 tumored hamster serum. Antigen is restricted to the nucleus and is chiefly in the form of rings, dots and flecks.

ceptible cells with inhibitors of DNA synthesis, cytosine arabinoside (Rapp *et al.*, 1965a) or fluorouracil deoxyriboside (FUDR) (Gilden *et al.*, 1965; Rowe *et al.*, 1965); treated cells show unimpaired formation of neoantigens, but marked inhibition or complete suppression of formation of the later viral antigens. It thus appears likely that neoantigens are formed by the DNA of the input virus while viral structural proteins require new DNA synthesis.

In preliminary FA studies with hamster tumors induced with Ad. 7, we have not found the consistency of pattern seen with Ad. 12. First, tumors induced by different strains of Ad. 7 differ markedly in content of FA-stainable antigen. Also, there is marked variation between sera of comparable CF reactivity, both with regard to per cent of tumor cells stained, ranging from 5–50% for reactive tumors, and apparently with regard to morphology of the antigen. In acutely infected tissue culture cells, we find that some hamster sera stain flecks, others stain inclusion-body type masses and others stain more amorphous material. It seems probable that Ad. 7 tumors differ in either the proportion of cells carrying the viral genome or in expression of the genome in synthesis of different antigens.

In addition to their value as specific diagnostic reagents for viral tumors and the insights into viral persistence in tumors and the early events in viral replication provided by neoantigen-reactive tumored hamster sera, these reagents have proved of great value in other areas. First, because of the high efficiency of neoantigen induction in the first cycle of infection we can determine the titer of adenoviruses with precision in a 1-day test simply by determining the proportion of cells showing specific nuclear staining at 21–24 hr; titers so obtained are within one-half to one log of the infectivity titer obtained a in 3–4 week test in the most sensitive cell system (Rowe *et al.*, 1965). Second, by the use of neoantigen-reactive sera it has been possible recently to demonstrate hybridization between unrelated DNA viruses, in that strains of several adenovirus types which had been grown with SV40 and freed of infectious

SV40 by antiserum are carrying the SV40 gene for neoantigen production within the adenovirus particle (Huebner *et al.*, 1964a; Rowe and Baum, 1964; Rapp *et al.*, 1964b). These adenoviruses produce tumors in hamsters which are primarily of SV40 histology (and serology); without neoantigen reagents this paradoxical observation could probably not have been clarified.

Application of the SV40 and Ad. 12 model (CF and FA reactivity between a tumor and late serum from the tumored host) to human tumors has given disappointingly negative results. Both Huebner (personal communication) and Rapp *et al.* (1965b) have studied large numbers of human tumors for autologous and homologous serum reactivity without convincing evidence of specific reaction. However, the variable of antibody response in the various model systems is so great that a negative result cannot be interpreted as indicating absence of persistent viral material.

In conclusion, I should like to express my admiration for the power and simplicity of the fluorescent antibody technique. Several years ago, I heard Dr. Coons preface an address on immunofluorescence with the apology that he is like a singer with a repertory of a single song. Even if this were true, which it is not, today's symposium has provided further proof that this song is like a Mozart aria, subject to countless beautiful variations.

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