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Heat-Labile Serum Factor Required for Immunofluorescence of Polyoma Tumor Antigens

Abstract. *The immunofluorescent demonstration of polyoma tumor antigens in tumor cells requires a heat-labile serum component. With unheated hamster tumor serum, specific fluorescence was observed in polyoma-transformed hamster, mouse, and rat tumor cells. Heated serum usually gave little or no reactivity; the activity of such heated serum could be restored simply by the addition of fresh normal unheated hamster serum.*

We have reported unsuccessful attempts to demonstrate polyoma tumor antigens (T-antigens) in hamster and mouse tumor cells by fluorescent antibody (FA) techniques (*1*). By the same methods, however, T-antigens were shown to be present in normal mouse embryo cells undergoing lytic polyoma infection. We have since found that polyoma T-antigens can indeed be detected in virus-free tumor cells by the FA procedure and that the reaction requires a heat-labile serum factor. These results are reported here.

In our previous experiments, we first tested tumor serums by the complement-fixation (CF) test to be certain that they contained antibodies against polyoma T-antigens. For the CF test, therefore, the serums were heated at 56°C for 20 min to inactivate complement. The negative results with immunofluorescence had thus been obtained with such heated serums. In reinvestigating the problem, fresh unheated polyoma hamster tumor serum was tested with a polyoma hamster tumor cell line designated as Py-T-54. The indirect method (*1*) was used. The unheated tumor serum gave bright intranuclear fluorescence (Fig. 1*A*). When the same serum was heated at 56°C for 20 minutes, fluorescence was almost totally abolished (Fig. 1*B*). With certain high-titer tumor serum (CF titer of 1/512), heating did not destroy all activity, but the pattern of fluorescence was quite different from that of unheated serum. Such heated serum gave a granular, discrete type of fluorescence (Fig. 1*C*). These results clearly indicated that a heat-labile serum factor was required for optimum fluorescence of polyoma T-antigens. To determine whether the factor was present in normal hamster serum, nonreactive heated tumor serum was mixed with an equal volume of fresh unheated normal hamster serum and tested for reactivity. Results of this experiment showed that unheated normal hamster serum contained the factor which completely restored reactivity of the heated tumor serum. Serum from eight individual adult hamsters were tested in a similar manner and all possessed the factor, indicating that a normal serum component was involved in the reaction. The normal serum could be diluted 1 : 8 without loss of activity. The effect of heating on other polyoma hamster tumor serums was also tested; in every instance, heated serum gave either a low degree of fluorescence or negative results. On the other hand, bright fluorescence was consistently found with unheated serum.

A number of other polyoma tumor cell lines transformed in vitro or in vivo were examined for T-antigens by immunofluorescence with unheated tumor serum (Table 1.) All cell lines tested were positive; however, the degree and type of fluorescence differed with the various transformed cells. On a roughly quantitative scale, the Py-T-54 (hamster) and the Py-1923 (mouse) cells gave the brightest fluorescence (+++). In general, there appeared to be a correlation between the CF titer of the various polyoma tumor cell lines and the degree of fluorescence observed. The Py-3T3-13 mouse cell line (received from G. Todaro), which was positive by the CF test only at undiluted cell concentration (10 percent cell suspension) was nevertheless positive by immunofluorescence, indicating a high degree of sensitivity of the test. Also of interest is the fact that an SV40-polyoma (SV-Py), doubly transformed, hamster cell line derived in this laboratory (*2*) contained both the polyoma and SV40 T-antigens by immunofluorescent as well as by CF tests. Among the cells tested, two lines (Py-T-59 and BHK-21, TC-1) were previously reported as negative when tested with heated tumor serum (*1*);

Table 1. Cells tested for immunofluorescence with unheated hamster polyoma tumor serum.

Cells tested	Species	CF titer*	FA	Source of cells
		Polyoma transformed hamster cells		
Py-T-54	Hamster	1/8	+++	LBV†
Py-T-59	Hamster	UND‡	+	LBV
BHK-21, TC-1	Hamster	1/4	++	M. Stoker
SV-Py	Hamster	1/4	++	LBV
Py-1923	Mouse	1/8	+++	LBV
Py-89	Mouse	1/2	+	R. Ting
Py-1498	Mouse	1/8	++	R. Ting
Py-3T3-13	Mouse	UND	+	G. Todaro
Py-3049	Rat	1/4	+	R. Ting
		Non-polyoma tumor cells		
SV 40-C11	Hamster	—	—	LBV
Rous tumor	Hamster	NT§	—	G. Rabotti
		Normal primary cells		
Hamster embryo		NT	—	
Mouse embryo		NT	—	
Rat embryo		NT	—	

* All cells tested as 10 percent frozen and thawed cell suspensions. † LBV, Laboratory of Biology of Viruses, National Institutes of Health. ‡ Und, positive undiluted only. § NT, not tested.

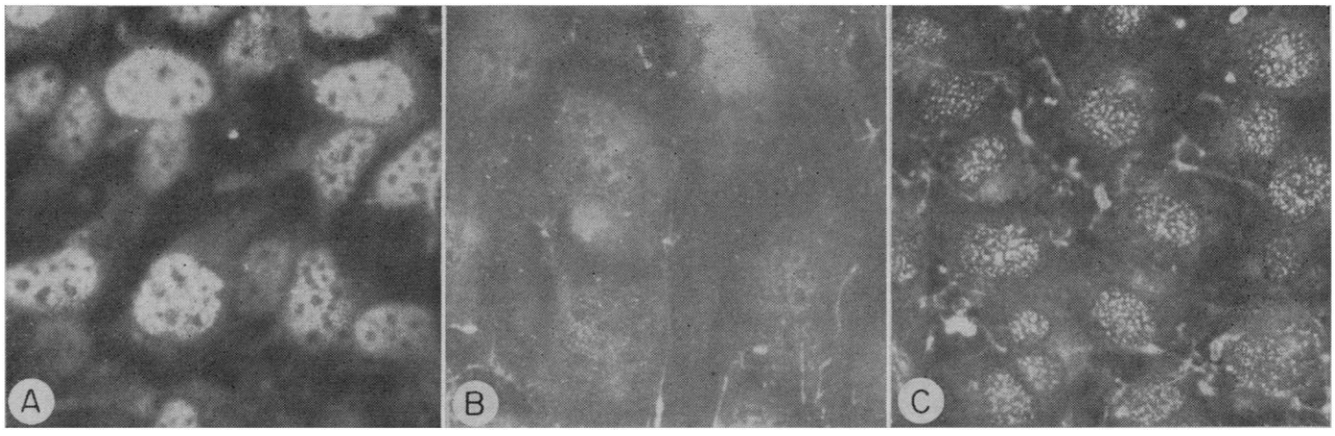

Fig. 1. Immunofluorescent reactions of Py-T-54 cells. *A*, Unheated, hamster polyoma tumor serum; *B*, same serum heated at 56°C for 20 minutes; *C*, heated, high-titer, hamster polyoma tumor serum (500 ×).

with unheated serum, these cells were now shown to be positive.

As controls, several different types of normal as well as tumor cells were tested with unheated polyoma tumor serum, and all were negative by the FA reaction. These controls included an SV40 hamster tumor cell line, a hamster tumor cell line induced by Rous sarcoma virus (this line was provided by G. Rabotti), and normal cells from the hamster, mouse, and rat embryo. In addition, unheated, SV40 tumor hamster serum was negative when tested with the Py-T-54 cells.

Attempts have been made to identify the heat-labile factor in hamster serum which enhances the reactivity of polyoma tumor serum with T-antigens. The heat lability of the factor and its presence in normal hamster serum was suggestive that it was complement. Experiments were therefore undertaken in order to establish the possible role of complement in the FA test. Fresh polyoma hamster tumor serum was heated at various temperatures up to 60°C for 20 minutes, then tested for both complement activity and ability to react with T-antigens by immunofluorescence using the Py-T-54 cells. Heating the serum at 50°C or higher removed all complement activity and also diminished fluorescence. At 56°C or 60°C most of the FA activity was lost. The thermolability of the serum factor thus appeared to coincide with that of complement.

Two other more specific experiments were done to test the hypothesis that the enhancing factor was complement (*4*). Since ethylenediamine tetraacetate (EDTA) prevents complement activity by chelating the necessary divalent cations, Ca and Mg, the FA reaction of unheated tumor serum with polyoma tumor cells was carried out in the presence of 0.01*M* EDTA. No effect on fluorescence was found by such treatment. In a second experiment, the complement activity of unheated tumor serum was removed by the addition of a precipitate of fetal bovine serum and rabbit antibody, with subsequent centrifugation at 600*g* for 20 minutes. Tests for residual complement activity showed that more than 90 percent of the complement had been removed by such treatment. The FA tests with this serum showed that its activity was essentially unchanged.

In other experiments, normal unheated serum from various species of animals was added to heated tumor serum to test ability to restore its activity. The indirect procedure with either fluorescein-conjugated antibody against the globulin of the species being tested or the conjugated antiserum to hamster globulin were tested. Negative results were obtained with guinea pig, rat, human, chicken, and monkey serums.

Although the foregoing experiments indicate a lack of relation between the enhancing factor and complement, further experiments are required to establish the identity of the factor. Payne *et al.* (*3*) have shown that a heat-labile factor was required for immunofluorescent reaction of chick embryo cells infected with Rous-associated virus and serum of hamsters bearing tumors induced by the Schmidt-Ruppin strain of Rous sarcoma virus. In their experiments, the reaction of heated serum could be restored by either normal hamster or guinea pig serum, suggesting that the serum factor was complement.

The FA technique provides a convenient and rapid method for studying polyoma T-antigens in both tumor cells and normal cells undergoing lytic infection. While the FA reaction of polyoma tumor serum with tumor cells requires a heat-labile serum factor, infected cells synthesizing the T-antigens do not need this factor since they can be stained with heated tumor serum. Our combined results (*1*) show that the polyoma tumor virus system bears close similarities to those described for the SV40 and oncogenic adenoviruses: the T-antigens which may be demonstrable by immunofluorescence as well as CF tests are present in cells that are infected and producing virus, and also in virus-free tumor cells. These antigens appear to be present in all cells transformed by the virus and apparently persist indefinitely.

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