Development of Antibodies Against Viral and Tumor Antigens of Papovavirus SV40 in Monkeys. (32207)

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(Introduced by J. L. Melnick)
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Antisera against papovavirus SV40 are usually prepared in monkeys(1-6). They are not only used for virus neutralization, but also as reference sera in the complement-fixing (CF) reaction(2,4,6) and in studies on virus reproduction by means of fluorescein-labelled(2,5,7,8) or CF antibodies. For these purposes antisera are necessary that contain only antibodies against the viral structural (V) antigen of SV40 but not against the new cellular antigens induced by the virus in the cells(2,9-15). One of these, denoted tumor (T) antigen, which is present in the nuclei of the SV40-transformed cells(14,15) is also synthesized in cells in which SV40 multiplies(3,16).

Huebner *et al*(2,17) reported that some of the SV40 antisera prepared in monkeys were CF-reactive with the T antigen prepared from SV40-transformed cells. At first these authors interpreted their finding as indicating a direct relationship between the V and T antigens(2); later they suggested that the respective monkey sera might contain two types of antibodies, one against the V antigen, the other against the T antigen (17). Sabin and Koch(3,18) examined a number of SV40 monkey sera from different laboratories, and concluded that the reaction of monkey sera with T antigen of SV40 was non-specific, although recently Geder and Sabin appear to have surmounted this problem(28).

The first data we obtained with SV40 monkey antiserum reacting with T antigen preparation were at variance with this conclusion. Therefore a study was undertaken to provide more information on the nature and the origin of the reacting substance.

Materials and methods. Cell cultures. Cell cultures from *Cercopithecus aethiops* monkey kidneys (GMK) were cultivated as described elsewhere(19). A continuous cell line (LA) derived from human amnion was cultivated in Parker's medium supplemented with 20% inactivated calf serum. Parker's medium with 5% calf serum was used as maintenance medium. A human diploid cell strain derived from human embryo lungs (LEP-14) was cultivated as described elsewhere(20).

The source of SV40 T antigen was the H-50 cell line developed by Ashkenazi and Melnick(1) and demonstrated to be virus-free(21). It was passed through hamsters and then carried in culture for several additional passages before use. The cells were grown in Parker's medium supplemented with the growth active alpha globulin of calf serum as recommended by Michl(22,23) and 10% calf serum. A cell line, TU-SP-2, was derived from a hamster tumor induced by the SP-2 stock of the SV40-adenovirus hybrid(24,25). The cultivation medium was the same as for the H-50 cells.

Viruses. SV40 and the SV40-adenovirus hybrid (SP2) were kindly supplied by Dr. Fred Rapp. The viruses were passed in GMK cells at an approximate multiplicity of infection of 0.1 to 0.01 PFU per cell. The viruses were harvested after complete degeneration of the cell sheet. The cultures were disrupted by 3 cycles of freezing and thawing and the fluid was clarified by low speed centrifugation. Until used, the viruses were kept at —20°C.

Adenovirus 7 prototype (Gomen) was obtained by courtesy of Dr. Brúčková. The virus was passed in LA cells.

Complement-fixing reaction. The drop technique on plexiglass plates was employed. The volume of each reagent was 0.03 ml. In the test 2-4 units of antigen, 1.7 units of complement and 10 units of hemolysin were used. The sera were diluted from 1:10 in 2-fold steps. The final concentration of sheep red blood cells was 0.06%. Complement was fixed for 16 hours at 4°C, after which sensitized red blood cells were added

and incubation was continued in a humidified box at 37°C for 2 hours. To enhance the clumping of non-hemolyzed cells, the plates were kept at 4°C for 15 minutes before being read. Hemolysis inhibition of 50% or more cells was considered a positive reaction.

Virus fluids prepared as described above were employed as SV40 V antigens. Only undiluted antigens not reacting with T antibodies (obtained from hamsters bearing tumors induced by SV40-transformed cells) were employed. Control antigens were prepared in a similar way from uninfected cultures. The SV40 T antigen was prepared from H-50 cell line of transformed cells. Cells grown in 1200 ml Roux bottles were washed with PBS (pH 7.3), trypsinized, centrifuged and resuspended in 10 volumes of PBS. After a single freezing and thawing, the suspension was used as CF antigen without further processing. Control antigen from primary uninfected hamster embryo fibroblasts was prepared in the same way. Adenovirus CF antigens were prepared from LA cells infected with the Gomen strain and from GMK cells infected with the SP-2 stock. Degenerated cultures were frozen and thawed repeatedly and the fluids were cleared by low speed centrifugation. Antigens not reacting with sera containing SV40 V and T antibodies were used. Corresponding control antigens were prepared from uninfected cultures.

The following sera were used as reference reagents: a) monkey SV40 serum selected for high CF antibody titer and giving no reaction with T antigen at a 1:10 dilution; b) negative monkey serum; c) serum pool of hamsters bearing tumors induced by H-50 cells with a high titer of T antibodies and not reacting with SV40 V antigen; d) negative hamster serum; e) human serum containing adeno CF antibodies; f) human serum without adeno CF antibodies.

Fluorescent antibody technique. The indirect technique was employed. Approximately 2×10^6 cells in 5 ml of medium were seeded into 60 mm Petri dishes containing cover slips. The cultures were incubated at 36°C in a humidified incubator containing approximately 5% CO_2 in air. When nearly complete cell sheets formed, the cultures were washed gently with maintenance medium and 2 ml of virus diluted 10^{-1} was added. After 1 hour incubation at 36°C, the unattached virus was rinsed off and 2 ml of maintenance medium was added. After 48 hours, the cover slip cultures were washed 3 times with PBS, left at 36°C for 30 minutes and then treated with acetone for 3 minutes. Thereupon, monkey serum was poured over the culture. Following 3 minutes incubation at room temperature, the antiserum was washed off with 3 changes of physiological saline and fluoroscein isothiocyanate-labelled rabbit anti-monkey globulin which had been treated with acetone-extracted mouse liver powder was added. The cultures were then washed as before and were mounted on slides in glycerol. They were examined with a Meopta microscope equipped with Zeiss Osram HBO-50 Mercury arc vapor lamp.

Neutralization test. The procedure employed was similar to that described by Ashkenazi and Melnick(1). The antibody titers were determined according to Kärber.

Immunization procedure. Cercopithecus monkeys weighing from 4 to 6 lb were used. Two monkeys (I and III) were inoculated intramuscularly with 1 ml of undiluted SV40 virus mixed with an equal amount of complete Freund's adjuvant. The other 2 monkeys (II and IV) were injected with SV40 virus without adjuvant. Blood samples were drawn before the inoculation and then at weekly intervals. Five weeks after the first injection, 1 ml of undiluted virus without adjuvant was administered intramuscularly to all 4 animals. Two additional blood samples were taken. The titer of virus inoculated was $10^{6.9}$ TCD_{50} per ml and the undiluted preparation did not react with SV40 T antibodies in the CF reaction. The same virus stock was used for both inoculations.

The sera were inactivated at 56°C for 30 minutes and were stored at —20°C until tested. All the sera were tested simultaneously.

Results. The CF results against the V and T antigens of SV40 are plotted in Fig. 1. It can be seen that all 4 monkeys developed

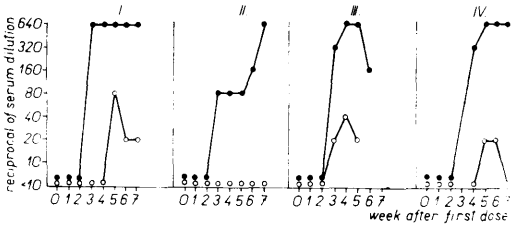

FIG. 1. Development of CF antibodies against V and T antigens of papovavirus SV40 in monkeys (●——● V antibodies, ○——○ T antibodies).

antibodies reacting with the V antigen. Antibodies were first detected 3 weeks after the monkey had been injected with virus. With the exception of monkey II the highest levels were reached before the second virus dose was administered. Three of the animals developed antibodies reacting with the T antigen; both animals which were injected with virus in mixture with adjuvant (I and III) responded. T antibodies were first detected in the 3-week sample of monkey III and in the 5-week samples of monkeys I and IV. The levels were lower and tended to decrease earlier than V antibodies. The second virus dose did not enhance T antibody levels in the 2 sera subsequently collected.

None of the preimmunization serum samples reacted with either the V or the T antigen preparation and none of the sera reacted with control antigen. The last 2 sera from monkey III were anticomplementary.

To corroborate the specificity of the reacting substance we used monkey antisera and the fluorescent antibody technique to localize the intranuclear T antigen in hamster cells transformed by SV40 or SP2 viruses and in monkey and human diploid cells infected with SP2 virus; only the T but not the V antigen of SV40 virus is formed in cells after SP2 infection(24,25). Two sera of monkey I were selected for the crucial experiment: serum No. 1 and serum No. 5. As has been shown above (Fig. 1), the first serum sample did not react with either of the antigens, while the fifth serum gave a positive reaction with both the V (1:640) and the T (1:80) antigens. Results of the fluorescent antibody tests are summarized in Table I. It can be seen that the first serum sample gave no staining in any of the cells. However, after treatment with the fifth serum sample, intranuclear fluorescence was observed in hamster cells transformed either by SV40 or SP2 virus. The antigen was also detected in both human and monkey cells which had been infected with SP2 virus, but not in the cells infected with the prototype adeno 7 strain. Monkey kidney cells infected with SP2 virus and treated with the fifth serum sample are shown in Fig. 2.

CF tests with adeno CF antigens did not reveal adenovirus antibody in either of the serum samples.

The neutralizing antibodies to SV40 were also measured in tests in which all the sera were tested simultaneously. The results of the tests are summarized in Fig. 3. All the animals developed neutralizing antibodies. It can be seen that their appearance preceded the CF V antibodies by one week. Otherwise the development of both neutralizing and CF antibodies followed the same pattern; this is most evident in monkey II, in which the second virus dose enhanced the antibody levels. In one of the animals (monkey IV), a low level of neutralizing antibody was detected in the preimmunization serum sample. This was the monkey that developed T antibody after having been injected with SV40 without adjuvant.

Discussion. The results presented here indi-

TABLE I. Immunofluorescence Tests with T Antigen in Various Cells Tested Against CF Negative and Positive Monkey Sera (Monkey I).

Cells	Immunofluorescence* 1st wk serum I/1 (CF neg)	5th wk serum I/5 (CF pos)
H-50 (transformed by SV40)	0	100
TU-SP-2 (transformed by SP-2)	0	100
Normal hamster fibroblasts	0	0
LEP-14 infected with:		
Adeno 7 (Gomen)	0	0
SP2	0	20
LEP-14 noninfected	0	0
GMK infected with:		
Adeno 7 (Gomen)	0	0
SP2	0	50
GMK noninfected	0	0

* Numbers represent approximate percentage of cells exhibiting intranuclear fluorescence.

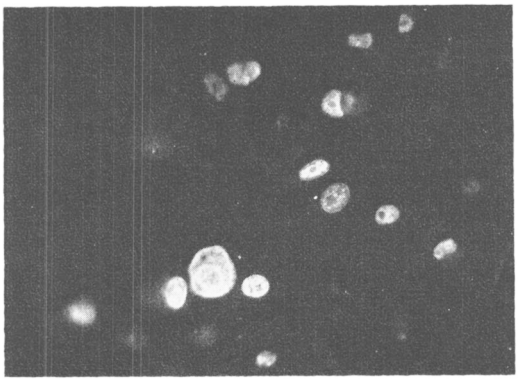

FIG. 2. Immunofluorescence micrograph of monkey kidney cells infected with SP2 virus and reacted with CF positive monkey serum (from 5th week bleeding of monkey I) 48 hr after infection. Stained with labelled rabbit anti-monkey globulin.

cate that antibodies against T antigen may develop in monkeys immunized with the SV40 virus. At variance with earlier reports, it seems clear that the reacting antibody is specific, a conclusion also recently reached by other workers(28,29). This conclusion is based on the CF results and is confirmed by the outcome of the fluorescent antibody tests with SV40 and SP2 virus-transformed hamster cells, and with the SP2-infected monkey and human cells. It had been shown(24,25) that the portion of SV40 genome encased in the adenovirus capsid in the SP2 virus population codes for the T but not the V antigen of the papovavirus. Because the monkey sera employed in the present work were free of adenovirus antibodies, the intranuclear fluorescence observed in SP2-infected cells must have been due to the reaction between the T antigen and the T antibody present in this serum.

Less clear is the origin of these antigens

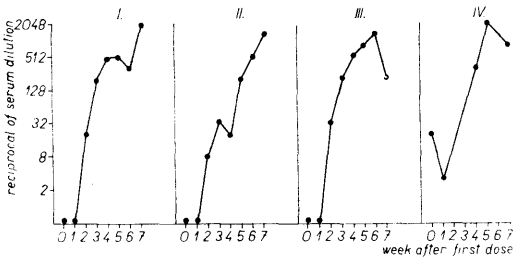

FIG. 3. Development of SV40 neutralizing antibodies in monkeys.

to which the animals responded. It is not known whether the monkeys responded to the T antigen present in the virus preparation or to that formed during the multiplication of the virus after its injection. The SV40 virus preparation used for inoculation was free of T antigen when tested by CF; however, it is possible though unlikely that a small amount of this substance was present in the virus preparation and was responsible for the antibody formation. It is obvious however that monkeys can be induced to synthesize T antibody. It is possible that they are more sensitive to the stimulation by T antigen than hamsters, in which T antibodies have been frequently detected but only after the development of large tumors (2,26,27).

Summary. Four monkeys were injected with undiluted papovavirus SV40; two of them received the virus with complete Freund's adjuvant. All 4 monkeys developed both complement-fixing and neutralizing antibodies against the structural antigens of the papovavirus. Antibodies against the T antigen induced by the virus were detected in the sera from 3 of the animals. The T antibodies appeared later, attained lower levels and tended to fall earlier than the V antibodies. The specificity of the substance reacting with the T antigen was demonstrated by immunofluorescence tests with cells transformed by SV40 virus and by the SV40-adenovirus hybrid (SP2) and with human and monkey cells infected with the SP2 virus.

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Production of Antibodies to Papovavirus SV40 Tumor Antigen in African Green Monkeys.* (32208)

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Simian papovavirus SV40 frequently produces latent subclinical infections in rhesus (1) and in African green monkeys(2,3). These animals respond to virus by producing viral neutralizing antibodies(2,3). Hamsters bearing solid tumors induced by the same virus develop an antibody which reacts specifically by complement fixation and immunofluorescence with an antigen (called tumor or T) present in the nucleus of all cells transformed by the virus either *in vitro* or *in vivo* (4-8). This antigen, though also induced during the replicative cycle of the virus(9-12), is not present in the intact virion.

This report describes the production of antibody against T antigen in African green monkeys upon inoculation of either SV40-infected African green monkey cells or extracts prepared from these infected cells.

Materials and methods. Virus. SV40 was the Baylor reference strain used in previous studies(9,13). Virus stocks were prepared in GMK cells as described previously (13).

Antigen preparation. Three types of preparations were used for inoculation of the monkeys. Primary green monkey kidney (GMK) cells growing in 16-oz bottles were inoculated with 5.7×10^5 plaque-forming units (PFU) of SV40. After one hour adsorption at 37°C, the cells were flooded with Melnick-Hanks' medium(14) and incubation continued at 37°C. Twenty-four hours later, the monolayers were washed with Tris buffered saline (TBS), pH 7.4. The cells were then scraped from the glass surface with a rubber policeman, sedimented and washed again with TBS. One preparation consisted of intact cells infected with the virus. A second preparation was prepared by sonicating the virus-infected cells for 30 seconds at 10 KC/second in a Raytheon sonic oscillator. The disrupted

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