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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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cells were then centrifuged and the supernatant was used to inoculate the monkeys.

A third preparation consisted of autologous cells inoculated with SV40. The left testicles of 3 African green monkeys were removed surgically and trypsinized according to the procedure outlined for monkey kidneys(14). The cells were resuspended in Eagle's medium supplemented with 10% autologous monkey serum and were seeded into 16-oz bottles. After a monolayer had formed, some of the cultures were inoculated with SV40 as described above. (The testicle cells in other bottles were dispersed with trypsin, resuspended in 5 ml of Eagle's medium containing 10% dimethylsulfoxide, sealed into ampoules, and stored in liquid nitrogen. These cells were then thawed and grown out for later inoculation of the autologous animal.) After the virus was inoculated, the testicle cells were incubated for 24 hours at 37°C in Eagle's medium containing 2% autologous monkey serum. Cells from a 16-oz bottle were then scraped from the glass with a rubber policeman, washed once with TBS, and suspended in 1 ml of TBS.

Animal experiments. Six African green monkeys *(Cercopithecus aethiops sabaeus)* were used in the study. All monkeys were bled prior to inoculation. Monkey GR-2721 received sonicated kidney cell material while the other 2 monkeys (GR-2723 and GR-2724) were inoculated with SV40-infected intact kidney cells. Each monkey was inoculated with 1 ml of the antigen preparation intramuscularly into the thigh for 14 consecutive days. Each dose represented material derived from one 16-oz bottle of SV40-infected GMK cells. The animals were again injected 28, 30, and 92 days after the initial inoculation of either the SV40-infected cells or their extracts. The monkeys were bled 3, 7, 10, 14, 17, 21, 28, 34, 92, and 99 days after the first inoculation. The sera were tested for the presence of complement-fixing (CF) antibodies against the T and viral (V) antigens. Sera from one of the animals was also tested for virus neutralizing antibodies.

The testicle cells were inoculated into the remaining testicle and scrotum of the monkey from which they had been originally obtained (GR-2749, GR-2759, GR-2762). These 3 animals were observed for possible tumors and bled periodically to test for evidence of complement-fixing antibodies to SV40 T and V antigens. Re-injection of animals was accomplished by retrieving cells from the frozen state and injecting them as described above.

Preparation of tumor and virus antigens. Tumor antigen for use in CF tests was prepared from hamster cells (H-50) transformed by SV40 *in vivo*(15). The cells are free of infectious virus(16) and do not synthesize the V antigen. Cells grown in 16-oz bottles in Eagle's medium supplemented with 10% calf serum were scraped from the glass surface with a rubber policeman and washed with TBS in the cold. The cells were diluted to make a 10% suspension and sonicated for 30 seconds at 10 KC/second. The supernatant after centrifugation was used as T antigen.

The V antigen used in the CF tests was the infectious virus and has been described (9).

Complement-fixation test. A micro-complement-fixation test was used to detect antibodies against T and V antigens. The details of the test as used in this laboratory have been published(17). Positive as well as negative controls were included with each test.

Neutralization test. Neutralization tests were performed on heated (56°C for 30 min) sera from monkey GR-2721 inoculated with SV40-infected GMK cell extracts. Various dilutions of antisera were incubated with 100 PFU of SV40 at room temperature for 1 hour. Infectivity was measured by the plaque method. Neutralization indices are expressed as the number of PFU that can be neutralized by 1 ml of the undiluted serum.

Results. The development of CF antibodies in monkey GR-2721 inoculated with extracts of SV40-infected cells is shown in Fig. 1. Antibodies to T antigen appeared as early as the 7th day after the initial inoculation; these antibodies were detected by the CF reaction as well as by immunofluorescence. The CF titer to T antigen reached a maximum of 160 on the 10th day and then dropped to 80 on the 14th day. The titers then remained constant through day 31.

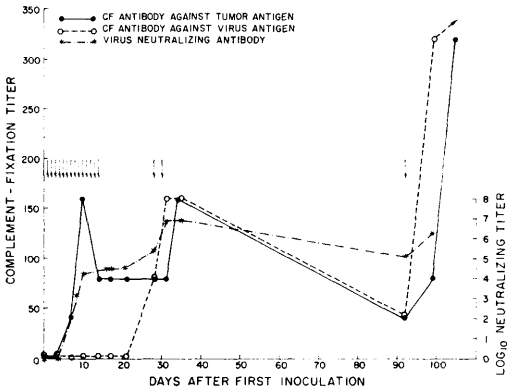

FIG. 1. Development of complement-fixing antibodies reacting with SV40 tumor (T) and virus (V) antigens and SV40 neutralizing antibodies following inoculation of an African green monkey (GR-2721) with extracts from green monkey kidney cells infected 24 hr previously with SV40. The monkey was inoculated daily for 14 days and then on days 28, 30, and 92 after the first inoculation.

Booster inoculations on days 28 and 30 elevated the titer on day 34 to 160; the titer then declined to 40 on day 92. After another booster on day 92, there was a rise in titer to 80 and 320 on the 99th and 105th day, respectively.

Complement-fixing antibodies to V antigen (Fig. 1) on the other hand did not appear until day 28. Similar to T antibody, the V antibody titer dropped to 40 on day 92 but increased to 320 by day 99 following the booster inoculation.

Neutralizing antibodies in monkey GR-2721 appeared by the 7th day after the initial inoculation and remained high throughout the experiment (Fig. 1). It is noteworthy that although the serum was capable of neutralizing more than 4 logs of virus (10-21 days), the CF test did not detect V antigen.

Antibody production against SV40 T and V antigens by monkeys inoculated with whole GMK cells infected with SV40 is shown in Table I (monkeys GR-2723 and GR-2724). In general, these monkeys did not respond as well as the monkey inoculated with the disrupted cells. As can be seen, antibodies against T antigen did not appear in monkey GR-2723 until sometime between the 17th and 34th day and monkey GR-2724 did not produce T antibody until day 99. The antibody titers of sera from monkey GR-2723 rose and declined as did those from monkey GR-2721.

The antibody responses of the monkeys inoculated with autologous testicle cells infected with SV40 are summarized in Table II. Two of the monkeys (GR-2759 and GR-2762) developed antibody to both the T and V antigen within 3-4 weeks after the initial inoculation. Although the titer against the T antigen decreased somewhat by 10 weeks after inoculation, a booster inoculation at 35 weeks restored the level to those previously observed. Monkey GR-2762 died 8 months after inoculation following an illness characterized by diarrhea and dehydration.

TABLE I. Development of Antibodies in Monkeys Following Inoculation* of Disrupted SV40-Infected Simian Kidney Cells.

Serum, days post-initial inoculation	Serologic results GR-2723 T	GR-2723 V	GR-2724 T	GR-2724 V
0	0	0	0	0
3	0	0	0	0
7	0	0	0	0
10	0	0	0	0
14	0	0	0	0
17	0	0	0	0
34	80	>320	0	40
92	10	40	0	0
99	80	>320	10	160
101	—	—	20	>320
104	160	>320	—	—

* Animals inoculated daily for 14 days, then on days 28, 30, and 92.
T = CF titer against T antigen.
V = CF titer against V antigen.

TABLE II. Development of Antibodies in Monkeys Following Inoculation* of Autologous Testicle Cells Infected with SV40.

Serum, weeks post-initial inoculation	Serologic results GR-2749 T	GR-2749 V	GR-2759 T	GR-2759 V	GR-2762 T	GR-2762 V
0	0	0	0	0	0	0
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	10	10
4	0	0	40	>80	10	40
5	0	0	>80	>80	10	>80
6	0	0	40	>80	20	>80
8	—	—	—	—	40	>80
10	—	—	20	>80	—	—
31	—	—	10	>160	—	—
35	0	10	—	—		
36	20	80	40	>160		
37	20	>160	20	>160		

* Animals inoculated on day 0 and 35 weeks after initial inoculation.

Monkey GR-2749 (Table II) failed to develop antibodies following the initial inoculation of the autologous cells. However, a booster inoculation elicited antibody response against both T and V antigens.

None of the animals observed developed tumors at the site of inoculation during the experimental period. Autopsy of the animal that died also failed to reveal evidence of neoplasia as determined by gross inspection.

Discussion. The studies reported here clearly demonstrate that African green monkeys synthesize CF antibodies to SV40 T antigen in addition to antibodies against the capsid (or V) antigen, in response to inoculation of either SV40-infected whole cells or their extracts. Antibody to T antigen in the monkey inoculated with cell extracts appeared as early as 7 days after the initial inoculation. This is in contrast to results obtained with monkeys inoculated with virus-infected whole cells (either autologous or heterologous) where the appearance of T antibody was not detected until between 17 and 34 days. It should be pointed out that some of the animals received inoculations each day for 14 days and were reimmunized again on days 28 and 30. Under these circumstances, there is constant antigenic stimulus similar to that in animals in which tumors are developing.

Previously, antibodies against SV40 T antigen have been reported to develop only in hamsters bearing tumors induced either by the virus or by cells transformed by SV40 (4-6). Preliminary observations(4,10,18) that had suggested the possibility of antibodies to T antigen developing in monkeys have been inconclusive because of non-specific reactions and difficulty in obtaining preparations of T antigen devoid of V antigen. These problems appear to have been overcome in later experiments by Geder and Sabin(19) as well as in the present study and that being reported by Vonka *et al*(20).

Our studies show that monkeys are capable of developing antibodies against SV40 T antigen even in the absence of a tumor. The reason that the T antibody can be readily detected in tumor-bearing hamsters is probably due to a continuous release of SV40 T antigen from the tumors which results in the development of a large quantity of antibody. This study revealed that the T antibody in monkeys can also reach a high concentration if sufficient antigenic stimulus is provided to the animals. This can be achieved by repeated inoculations of T antigen, or by inoculation of virus-infected cells (which are also accompanied by infectious virus). The development of T antibody in monkeys in the absence of a tumor also indicates that T antigen is not invariably associated with viral carcinogenesis *in vivo*.

Summary. African green monkeys repeatedly inoculated with intact simian cells infected with simian papovavirus SV40 or with disrupted infected cells developed complement-fixing antibodies capable of reacting with the virus tumor and virus capsid antigens. The antibody against the tumor antigen appeared first and titers could be maintained or increased by reimmunization of the animals. In addition, virus-neutralizing antibodies were detected. These results demonstrate that antibody against the tumor antigen of the simian papovavirus, an antibody with the same specificity as that which appears in tumor-bearing hamsters, can be elicited in primates in the absence of detectable tumors.

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A New Diuretic Drug Dependent on the Adrenal for its Action. (32209)

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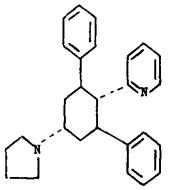
In chemical studies of compounds related to lobinaline, Robinson and coworkers(1) of this laboratory synthesized the following substance:



2-[2,6-Diphenyl-4(1-pyrrolidinyl) cyclohexyl]-pyridine

(Su-15049)

Its primary pharmacological action in rats is reported here. It was to induce a natriuresis and water diuresis with little or no kaliuresis. Similar actions in dogs have been shown by Barrett *et al*(6) and Cohen and Cafruny(7).

Methods. Unless otherwise stated, male rats (180-200 g) were fasted overnight but allowed water and then given 0.2% NaCl, 5 ml/100 g, by stomach tube. The animals were then placed in individual metabolism cages and urinary volumes recorded every 30 minutes for 3 hours. Sodium and potassium content of the total urine collections was measured at the end of 3 hours by flame photometry.

Su-15049† was suspended in a vehicle of carboxymethylcellulose and administered by stomach tube at the time of fluid loading. The steroids used were similarly suspended except for the low doses (0.2 and 10 μg) of aldosterone which were in aqueous solution. The steroids were given subcutaneously one hour before fluid loading and dosages indicated in Table I are per rat. Control groups, either intact or adrenalectomized, received the appropriate vehicle or solvent of the drug being tested.

In most of the groups shown in Table I, in which individual drugs or treatments were being compared, equal numbers of treated and control animals were included in each day's run. Series 1, on the other hand, is made up largely of a pool of control animals

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† The water-soluble citrate of Su-15049 (designated Su-15049A) is being used by most other groups studying this compound because of its greater convenience. With regard to sodium excretion, the dose-response curve of Su-15049A in rats is identical to that of Su-15049 (Fig. 1), when given in equimolar amounts of base. A slight enhancement of potassium excretion was not of proven signficance.