Sex-related Resistance in Hamsters to Adenovirus-12 Oncogenesis

II. Influence of Virus Dose

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A significantly higher proportion of female hamsters developed tumors than did males given the same dose of adenovirus-12 (Huie) at birth over a dose range from $10^{5.0}$ to $10^{7.0}$ TCD₅₀ for human embryonic kidney cells. The 50% tumor dose (TD₅₀) was calculated to be $10^{5.90}$ TCD₅₀ for females and $10^{6.27}$ for males. Tumor response patterns induced with approximate TD₅₀ inocula, $10^{6.0}$ for females and $10^{6.3}$ for males, were quite similar. The greater susceptibility of females was not found to be characteristic of a single strain of hamsters; nor was it attributable to a single lot of virus, to a single type of human cell used to produce the virus, nor to the degree of purification of the virus inoculum. The inoculation route did not appear to be of importance. Inasmuch as the foregoing extrinsic factors were of little influence, it was concluded that the mechanism is host-mediated, presumably hormonally controlled. The possibility that female cells, independent of host control, are more susceptible to adenovirus-12 oncogenesis than male cells has not been explored. Tumor regression occurred in 20% of the 211 tumors in males and in 15% of the 355 tumors in females. Adenovirus-12 T-antibody was detected in all but six of 473 sera tested from tumor-bearing hamsters and in 50% of 94 sera tested from non-tumor-bearing animals given virus at birth. Antibodies in the latter group were detected almost exclusively by indirect immunofluorescence. This technique appears to be extremely sensitive for detection of low levels of adeno-12 T-antibodies. The implications of T-antibody in nontumor-bearing virus-injected hamsters are discussed. Sera from normal hamsters were free of T-antibody.

Previously, we reported that male Syrian hamsters (*Mesocricetus auratus*) were more resistant to adenovirus-12 oncogenesis than were females (15). A similar observation has been reported by McFarlane and Embil (5) and personally communicated by John Trentin. In addition, Robert Hull (*personal communication*) has noted a similar situation with the oncogenic simian adenovirus, SV-20, but not with SA-7. Suggested explanations have attributed the difference to hormonal influence, karyologic differences between sexes, peculiarities of the virus preparations and dose, and properties of the hamster strain. This report describes studies which indicate that the resistance is not associated with a single strain of hamsters and is independent of virus dose and virus production methods.

MATERIALS AND METHODS

Virus. Adenovirus-12 seed stock of prototype strain Huie was supplied by Robert Huebner of the National

Institutes of Health. Initial passages and pools were made in HeLa cells free of pleuropneumonia-like organisms. For experimental purposes, certain virus pools were prepared in primary human embryonic kidney (HEK) cultures. Passage history is shown in Table 1. HeLa cells and primary HEK cells were grown in Eagle's basal medium containing 10% agamma or fetal calf serum. Virus pools were produced in cultures maintained with Eagle's basal medium or medium 199 containing 2% fetal calf serum. Certain virus pools were concentrated and then purified by CsCl density gradient techniques. Duplicate titrations of three or more samples from each pool were performed in primary cultures of HEK. Titers were calculated according to the method of Reed and Muench (10) and expressed in TCD₅₀ per unit volume; titers shown represent mean titers of the replicate titrations.

Hamsters. Pregnant, random-bred Syrian hamsters (*Mesocricetus auratus*) were obtained from Con Olson Hamstery of Milwaukee, Wis. Pregnant inbred Syrian hamsters, line PD-4, were obtained from Rae Whitney, of Horseheads, N.Y. The latter hamsters have been brother-sister mated for more than 40 generations.

Within 24 hr after birth, 0.1 ml of virus was injected into the dorsal subcutaneous tissue of the newborn hamsters. All hamsters were weaned at 3 weeks of age and segregated according to sex. No experimental treatment was given the animals included in this report. The animals were palpated 2 or 3 times weekly for tumor until 4 months of age; at this time, observations were terminated and the animals sacrificed.

Segments of each tumor were taken at autopsy and fixed in Lillie's fixative (acetic acid, Formalin, and alcohol). Histological examination was made of essentially one-third of the tumors.

Serology. Sera were collected from all animals when they were sacrificed. Whenever a tumor regressed, a trial bleeding was taken 2 to 4 weeks thereafter. All sera were tested either at a 1:10 dilution by complement fixation (CF) or at 1:3 dilution by indirect immunofluorescence (IF) for adeno-12 T-antibodies. The CF procedure, a tube test with 1.5 total volume, was performed as described previously (15).

The indirect IF test was performed with hamster sera diluted 1:3 in phosphate-buffered saline containing 5% bovine albumin plus 1 mg/ml of rhodamine-labeled bovine albumin. The secondary reagent was rhesus monkey anti-hamster γ -globulin labeled with fluorescein isothiocyanate according to the method of Riggs et al. (11) and absorbed with hamster adeno-12 tumor. BHK-21, HEP-2, or AV-3 cells were grown in EBM on cover slips in Leighton tubes. Adenovirus-12 was added at a multiplicity of 1 to 2 TCD₅₀ per cell.

When HEP-2 or AV-3 cells were employed, arabinofuranosyl-cytosine was added to a concentration of 15 μ g/ml, 12 to 14 hr after infection. After a total of 36 to 40 hr of incubation at 37 C, the cover slips were rinsed in phosphate-buffered saline and fixed with 1% neutral Formalin for 20 min. They were then air-dried and fixed with cold acetone for 10 min. Primary reagents were added and incubated 60 min at 37 C. The cover slips were rinsed in phosphate-buffered saline, and the secondary reagent, which had been diluted in phosphate-buffered saline to contain 0.8 mg of globulin per ml, was added and the cover slips were incubated at 37 C for 45 min. Cover slips were mounted in buffered glycerol and examined by dark-field microscopy with ultraviolet illumination. The filter system was a UG-1 or UG-5 exciter filter combined with a 430-m μ barrier filter. Cells were examined for immunofluorescence of the intranuclear flecks [bundles of fibers (3)] which are pathognomic of adenovirus-12 T-antigen (3, 4, 8). Appropriate blocking and known positive and negative controls were included.

RESULTS

Influence of virus dose on the differences in tumor responses between sexes. Table 1 lists, among other data, tumor incidence at 120 days after subcutaneous injection of various doses of adenovirus-12 in newborn hamsters. In normal hamsters, whether random bred or inbred, tumor incidence

TABLE 1. Adenovirus-12 passage history, virus dose injected, and tumor incidence by sex in non-inbred and inbred Syrian hamsters

Log ₁₀ TCD ₅₀ injected	Virus grown in	Passage no. and pool letter	Hamster breed	Tumor incidence and per cent at 120 days	
				Males	Females
5.0	HeLa	4-E	Noninbred	1/8 (12.5)	4/13 (31)
5.2	HeLa	6-K	Noninbred	0/5	4/13 (35)
			Inbred	1/10 (7)	2/4
5.7	HeLa	3-C	Noninbred	2/20 (10)	18/35 (51)
6.0	HeLa	1-A	Noninbred	7/31 (23)	20/36 (56)
	HeLa	2-B	Noninbred	7/27 (26)	18/33 (55)
	HeLa	6-P, Q	Noninbred	8/22 (36)	11/16 (69)
			Inbred	21/54 (39)	28/50 (56)
			Total	43/134 (32) ^b	77/135 (57) ^b
6.3	HEK	7-S	Inbred	30/63 (48)	53/70 (75)
	HEK	7-S	Inbred	51/87 (59)	62/88 (70)
	HEK	7-S	Inbred	26/61 (43)	39/60 (65)
			Total	107/211 (51) ^b	154/218 (70) ^b
6.7 ^a	HEK	6-I	Noninbred	34/44 (77)	47/51 (93)
7.0 ^a	HeLa	6-J	Noninbred	23/28 (82)	33/34 (97)

^a Pools I and J were concentrated and purified by density gradient centrifugation in CsCl and diluted to yield 10^{6.7} and 10^{7.0} TCD₅₀, respectively, per 0.1 ml.

^b Represents points through which lines in Fig. 1 are drawn.

was always higher in female hamsters than in male hamsters with virus doses from $10^{5.0}$ to $10^{7.0}$ TCD₅₀ (as titrated in human embryonic kidney cells). That this consistent observation in 12 experimental groups was due to chance, had a probability value of <0.001 as determined by:

$$\chi^2 = \frac{(P - N)^2}{P + N},$$

where P = the number of groups with this observation and N = the number without this observation.

In order to visualize the relationship between virus dose and tumor incidence, the overall tumor incidence by sex for 259 hamsters (134 males and 135 females) given $10^{6.0}$ TCD₅₀ and 429 hamsters (211 males and 218 females) given $10^{6.3}$ TCD₅₀ was plotted against $\log_{10}$ of virus dose on normal probability paper (Fig. 1). The line through these points was extrapolated to generate expected tumor incidences at other doses. This procedure was based on the expectation that the responses would follow the S-shaped normal dose response curve. Observed values with other virus doses were plotted in Fig. 1. Observed values were compared with expected values predicted by Fig. 1. The majority of observed tumor incidences plotted, $2\frac{1}{2}\%$ or 91%, fell within expected values ± 2 standard deviations. Furthermore, 54.5% or $\frac{1}{2}$ observations in each sex group fell within ± 1 standard deviation of expected

values. These results suggest that the observed tumor incidences followed a normal distribution pattern and that the expected values generated by the lines in Fig. 1 can be used to estimate the 50% tumor dose (TD₅₀) for each sex. These values are $10^{6.27}$ for males and $10^{5.90}$ for female hamsters. The difference, $10^{0.37}$, indicates that approximately 2.3 times more virus is required in males than in females, to achieve the same tumor incidence.

It is noted that the tumor response patterns of males given $10^{6.3}$ TCD₅₀ was very similar to that of females given $10^{6.0}$ TCD₅₀ (Fig. 2). This indicates that the distinguishing differences in male and female tumor response patterns are greatly diminished at their approximate respective TD₅₀ levels.

Tumor formation was detected earlier in females than in males. Actually, no palpable tumors were found in males prior to the 28th day postinjection, whereas in females given $10^{6.7}$ TCD₅₀, tumors developed by day 25. With hamsters given $10^{6.3}$ TCD₅₀, the tumor incidence by day 30 was 1% in males and 13% in females. After the same interval, with doses of $10^{6.7}$ and $10^{7.0}$ TCD₅₀, the tumor incidence in males was 14 and 18%, respectively, as compared to incidences of 49 and 23% in females. The rate at which tumors appeared was more rapid in females (Fig. 2). This difference was more marked with

FIG. 1. Adenovirus-12 tumor dose response relationship in hamsters.

FIG. 2. Influence of dose of adenovirus-12 on tumor incidence and rate of tumor appearance in Syrian hamsters.

lower virus doses, $10^{6.0}$ TCD₅₀ or less, than with higher doses.

Influence of other extrinsic factors on tumor responses. Various other extrinsic factors associated with the virus inoculum were not as extensively explored as was virus dose. However, the data shown in Table 1 reveal that the difference in tumor incidence between sexes at 120 days was (i) maintained through seven serial passages of the virus in this laboratory, (ii) apparent whether the virus was produced in HeLa or primary HEK cells, and (iii) present regardless of the relative purity of the virus. In regard to the latter factor, two pools, I and J, grown in HEK and HeLa, respectively, at the 6th passage level were concentrated and purified by banding in CsCl at density 1.34. The banded virus was diluted in phosphate-buffered saline to yield the concentrations shown in Table 1. These two purified pools represented the two highest concentrations of virus employed in these studies; both yielded higher tumor incidences in females than in males.

One other extrinsic factor, the route of virus injection, may require examination. However, some data on normal hamsters given 0.05 ml ($10^{7.7}$ TCD₅₀) of purified adenovirus-12 intravenously at birth (via the patent umbilical vein) indicate that tumor incidence among hamsters sacrificed from the 16th to the 22nd day post-inoculation, was slightly higher in females (experiment 23, Table 2). In another experiment, to be described in detail elsewhere, 100% of 16 animals given $10^{8.0}$ TCD₅₀ intravenously at birth produced adeno-12 tumors within 31 days, re-

gardless of sex. However, the animals in this latter study (experiment 24, Table 2) were sacrificed when moribund due to liver tumors. The data suggest more rapid development of tumors in females than in males.

Serological studies. Serum was collected from 473 of the 566 hamsters which developed tumors (Table 3). No serum was obtained from 93 hamsters, owing to unexpected death of the animals or other technical reasons. Tumors regressed in 94 of the 566 hamsters and a serum sample was collected from 89 of the 94 within 2 to 4 weeks following tumor regression. The regression rate was 20% in males and 15% in females.

Serum was also collected from hamsters which did not develop detectable tumors by 120 days postinoculation. Serological tests were conducted on 94 of these sera.

Generally, a 1:10 dilution of each serum was tested by CF. All negative sera, anticomplementary sera, and questionable or weakly reactive sera, plus a number of strongly reactive sera, were tested at a 1:3 dilution by IF. In addition 92 sera from the tumor groups were tested by IF alone.

Of the 384 sera tested by one or both tests from hamsters with tumors which did not regress, only two nonreactive sera were found. The intervals between tumor appearance and serum collection from these two hamsters were 11 and 28 days, respectively. The 11-day interval may have been too short for antibody to have accumulated to detectable levels. The reason for absence of detectable antibody in the hamster with tumor for 28 days is not readily apparent. Tumors from

TABLE 2. Tumor incidence in PD-4 hamsters injected intravenously at birth and sacrificed at various times thereafter

Expt no.	Virus pool and passage	Log ₁₀ TCD ₅₀ injected	Days after intravenous injection ^a	Tumor-bearing animals/no. of animals sacrificed	
				Males	Females
23 ^b	X-7	7.7	16	1/5	0/5
			18	0/6	1/2
			20	1/8	3/7
			22	2/8	1/7
			Total	4/27 (15%)	5/21 (24%)
24 ^c	Y-7	8.0	12	—	1/1
			18	—	1/1
			24	—	5/5
			27	2/2	2/2
			29	2/2	1/1
			31	2/2	—
			Total	6/6 (100%)	10/10 (100%)

^a Animals injected within 8 hr of birth with 0.05 ml of purified adenovirus via jugular vein or patent umbilical vein.

^b Animals in experiment 23 were sacrificed at random according to the times listed.

^c Animals in experiment 24 were sacrificed when obviously moribund with tumor.

TABLE 3. *Complement-fixation and indirect immunofluorescence tests on sera from tumor-bearing, nontumor-bearing, and normal hamsters*

Hamster group	Sex	Total animals	Sera tested	No. positive/no. tested and per cent positive by		
				CF ^a	IF ^b	CF and/or IF
With sustained tumors	♂	169	160	114/125 (92)	46/48 (96)	158/160 (99)
	♀	303	224	178/180 (99)	70/70 (100)	224/224 (100)
	Total	472	384	292/305 (96)	116/118 (98)	382/384 (99)
With regressed tumors	♂	42	41	14/36 (40)	38/41 (93)	38/41 (93)
	♀	52	48	15/40 (38)	40/41 (98)	47/48 (98)
	Total	94	89	29/76 (39)	78/82 (95)	85/89 (97)
Received virus but no tumor detected ^c	♂	252	59	1/59 (2)	30/59 (51)	30/59 (51)
	♀	164	35	1/35 (3)	17/35 (49)	17/35 (49)
	Total	416	94	2/94 (2)	47/94 (50)	47/94 (50)
Not given virus	♂	30	30	0/30 (0)	0/30 (0)	0/30 (0)
	♀	30	30	0/30 (0)	0/30 (0)	0/30 (0)
	Total	60	60	0/60 (0)	0/60 (0)	0/60 (0)

^a Complement fixation (CF) tests were performed with 1:10 dilution of serum.

^b Immunofluorescence tests (IF) were performed with 1:3 dilution of serum.

^c Animals sacrificed at 120 days of age.

both animals resembled typical adeno-12 tumors grossly and microscopically as described by others (1, 2, 6, 7, 9, 12-14).

Of the 89 sera tested from the 94 hamsters in which tumors regressed, all but 4 were reactive by IF, whereas only 29 of the 76 sera tested by CF from this group were positive. This indicates that the indirect IF test is a highly sensitive method for detecting low levels of adenovirus-12 T-antibodies. Rapp et al. (9) have reported a similar experience.

A further example of the IF sensitivity is apparent from the serological tests on sera from hamsters which, although given virus at birth, did not develop tumors. T-antibodies were detected at 1:3 dilution by indirect IF in 47 sera of 94 tested; only 2 of these sera were positive by CF at the 1:10 dilution. The IF reactions with these sera were less intense than with the CF-positive sera from tumor-bearing hamsters, but were similar in intensity to reactions obtained with CF-negative sera from tumor-bearing hamsters. The distinctive IF staining pattern pathognomic for adenovirus T-antigen (3, 4, 8) was readily obtained with all sera designated as IF-positive. All sera tested from animals not given adenovirus-12 virus were consistently nonreactive by either test. The presence of T-antibodies in 50% of the sera from virus-injected animals that did not form detectable tumors indicates that T-antigen was synthesized in immunologically stimulatory amounts in these animals.

DISCUSSION

These studies have extended and confirmed our original observation that a greater proportion of female hamsters develop tumors when inoculated subcutaneously at birth with adenovirus-12 than do male hamsters (15). This observation has been a consistent finding over a 100-fold differential in virus inoculum, i.e., from $10^{5.0}$ to $10^{7.0}$ TCD₅₀. The greater susceptibility of female hamsters is generally characterized by an earlier development of tumors and a more rapid appearance rate than in males; these differences are somewhat more pronounced with lower virus doses.

These studies have also established that the difference in susceptibility (or resistance) between sexes is not restricted to a single source of hamsters; both noninbred golden Syrian hamsters and inbred white Syrian hamsters from two different suppliers yielded similar observations. In this regard, the reports (5; J. J. Trentin, *personal communication*) confirming our observation were obtained with hamsters from still other suppliers.

The cell type employed to produce the virus and the relative purity of the viral inocula do not seem to be important factors. Virus grown in HeLa or HEK cells yielded similar findings. Removal of the media and cellular components from the virus preparation by CsCl banding of the virus did not alter the susceptibility difference. The crude virus pools were free of pleuropneumonia-like organisms, as determined by *in vitro*

culturing techniques, and were apparently free of adeno-associated virus as determined by electron microscopic examination of infected cells and of pelletized virus.

The influence of the route of injection (14) was not explored extensively in these studies. However, the communication from Trentin indicates that the difference is apparent regardless of route. Our results with intravenous injection are also indicative that the route of injection is not of major importance.

Since it seems that the above-cited extrinsic factors play little or no apparent role in inducing higher tumor incidences in female hamsters than in males, it appears that the determining mechanism is host-mediated. This conclusion has incited extensive investigations of the influences of sex hormones on adenovirus-12 oncogenesis; these studies will be reported in detail elsewhere. A preliminary report (Yohn et al., *Proc. Am. Assoc. Cancer Res.* 6:70, 1965) that ovariectomy at 3 weeks of age reduced tumor incidence in females, whereas castration of males was without influence, exemplifies the likely role that estrogens play in the mechanism. It has also been previously reported that thymectomy (15) but not splenectomy (5) at 3 weeks of age enhanced the tumor response in males but not in females. These observations seem to indicate that hormonal influences on immunological development or reactivity in the suckling or weanling hamster may be primarily responsible for the greater susceptibility of female hamsters to adenovirus-12 oncogenesis. The lower proportion of tumor regressions in females is probably another manifestation of the mechanism which yields higher tumor incidence in females.

Although the observation that slightly more than twice (2.3 times) as much virus was required in males to achieve a 50% tumor incidence may be interpreted to indicate that female hamster cells per se are more susceptible to adenovirus oncogenesis, a possibility yet to be explored, it seems more likely that the mechanism(s) responsible for the greater susceptibility of females function only in vivo and are, therefore, probably linked to the differences between male and female hormonal influences on cell growth and immunological reactivity.

The detection of adeno-12 T-antibodies by immunofluorescence in 50% of the sera tested from nontumor-bearing hamsters, sacrificed 120 days after receiving adeno-12 virus at birth, was of interest. This can best be interpreted to indicate that T-antigen is synthesized in most hamsters in amounts sufficient to induce an

immunological response. It cannot be stated with complete certainty that the only source of the synthesis of this amount of antigen would be in adenovirus-12 neoplastic cells, but this must be considered the most likely explanation. That frank palpable tumors actually regressed in 94 hamsters with resultant sero-conversion in 97% of them, is direct evidence that neoplastic cells do form but, subject to the immunological capacity of the host, may be rejected. Thus, it seems justified to believe that the cell-virus interactions necessary for tumor formation occur in all hamsters given a certain minimal dose of adenovirus-12 at birth and that neoplastic cells develop. The initial factors which determine their fate are probably those factors that influence cellular replication rates such as nutrition, blood supply, or host-mediated growth-controlling factors (hormones). Later, immunological mechanisms exert their influence. It was shown herein that tumor regression, presumably immunologically mediated, occurred after tumors developed. It is quite likely that immunological rejection of neoplastic cells occurs in some animals prior to the replication of sufficient tumor cells which can be located by simple palpation. We believe it is this latter circumstance which results in the production of T-antibody by nontumor-bearing hamsters.

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