

INDUCTION OF LEUKEMIA IN RAT BY PULSE DOSES OF 7,12-DIMETHYLBENZ(a)ANTHRACENE*

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Under simple conditions, an intravenous injection (pulse dose) of large but tolerable amounts of certain polynuclear aromatic hydrocarbons evokes mammary cancer in every rat,¹ whereas leukemia arises rarely. In the present work, it was found that by rearrangement of the experimental conditions, leukosis and leukemia were induced in high yield but with small incidence of cancer of the breast.

In rat, a pulse dose of 7,12-dimethylbenz(a)anthracene damages only those cells which synthesize DNA,² whereas cells which proliferate by meiosis are not injured.^{3, 4} A single pulse dose of 7,12-DMBA effectively induces mammary cancer under stated conditions. By definition, an effective amount of 7,12-DMBA is that quantity which induces mammary cancer in every rat but is not lethal to any animal. Three equal fractions of the largest tolerable and effective amount given at intervals of 3 days called forth more cancers than the total amount injected on one occasion;⁵ each of the fractions itself evoked cancers in every animal.

The leukemogenic action of hydrocarbons was discovered by Morton and Mider⁶ by repeatedly painting the skin of mice with 3-methylcholanthrene. The incidence⁷ of leukemia was 99 per cent with mean latent period of 86 days; in the predominant type of leukemia there was enlargement of thymus, liver, spleen, and lymph nodes.

In the rat, prolonged feeding of aromatic hydrocarbons elicited leukemia in rather low yields. The incidence of leukemia evoked by various hydrocarbons in feeding experiments was 3-MC (ref. 8), 14 per cent; 7,12-DMBA (ref. 9), 47 per cent; 2-acetylaminophenanthrene (ref. 10), 48 per cent.

Experimental.—Normal rats of Long-Evans strain, bred at random, were used exclusively. They were provided a commercial ration and water *ad libitum* and kept in air-conditioned rooms at $25^{\circ} \pm 1$. A lipide emulsion¹¹ of 7,12-DMBA (0.5% w/w) was injected in a caudal vein. No single injection exceeded 6 mg of 7,12-DMBA. The day of the first pulse dose is designated day 0. The rats were weighed thrice weekly.

Leucocyte count, differential cell count, and hemoglobin were determined in heparinized venous blood. Components of the buffy coat were measured in a slender glass capillary tube (75 mm in length; 0.3 mm internal diameter; wall thickness 0.2 mm) which was filled with blood and centrifuged at 12,000 rpm for 5 min; the thickness of the layers of the buffy coat was measured immediately by microscopy using an ocular micrometer; the results are expressed in percentage of the length of this microhematocrit.

Surgical operations were performed under ether anesthesia. Prior to the first pulse dose of 7,12-DMBA, in some experiments, spleen was exteriorized to a subcutaneous position, leaving its vascular pedicle intact. Biopsy of liver was done through an abdominal incision, 1 cm in length, and a wedge (*ca.* 50 mg) of the periphery of the organ was excised for histologic study.

TABLE 1
LEUKEMIA EVOKED BY SINGLE OR MULTIPLE PULSE DOSES OF 7,12-DMBA

No.	Injections of 7,12-DMBA		No. rats	Leukemia	
	Age, days	mg/kg		No.	%
1	50	40	48	3	6.3
3	50; 60; 70	40; 30; 30	34	25	73.5

Female rats were injected i.v. and observed for 270 days.

Whole blood, 0.2 ml, was injected i.p. in newborn rats. Blood plasma was similarly injected after filtration through Millipore filter with pore size 0.45 μ ; the filters were impermeable to *E. coli* added to the plasma.

Results.—Very few spontaneous tumors have been observed in our colony of Long-Evans rats, mostly benign tumors of the mammary gland. Leukemia was detected in only one animal among 2,000 untreated rats age 6+ months; the leukemia was of thymic type.

Tolerance of pulse doses of 7,12-DMBA: Young rats are more tolerant of 7,12-DMBA than adult rats. LD₅₀ for Long-Evans rats was determined by the probit method¹² after a single i.v. injection of 7,12-DMBA. At age 25 days, LD₅₀ was 73 mg/kg; at age 40 and 100 days, LD₅₀ was 44 mg/kg. No fatality followed pulse doses of 7,12-DMBA, 50 mg/kg at 27 days or 35 mg/kg at 40–100 days.

Incidence of leukemia following pulse doses of 7,12-DMBA: Forty-four female rats were given pulse doses of 7,12-DMBA, 16 mg/kg, at age 50 and 53 and 56 days, and were observed for 7 months. The incidence of tumors was leukemia, 11 per cent; mammary cancer, 36 per cent. Sprague-Dawley rats treated in a similar manner have 100 per cent incidence⁵ of mammary cancer.

Female rats were injected with a single pulse dose of 7,12-DMBA, 40 mg/kg at age 50 days; the incidence of leukemia was 6.3 per cent (Table 1). Companions were given three injections of 7,12-DMBA at age 50, 60, and 70 days; the incidence of leukemia was 73.5 per cent; mammary cancer developed in 38 per cent.

Groups of male and female rats were given four pulse doses of 7,12-DMBA at biweekly intervals beginning at age 27 days. Every rat developed leukemia (Fig. 1); the animals were sacrificed in the terminal stage of the disease. Survival of the leukemic rats after the first injections of 7,12-DMBA was males 132 \pm 59 days, females 112 \pm 37 days. Mammary cancer was observed in 12.5 per cent of the animals of each sex.

Forty-four male rats were given seven pulse doses of 7,12-DMBA at biweekly intervals beginning at age 27 days. Every rat developed leukemia (Fig. 2); the mean survival was 102 \pm 22 days; mammary cancer was detected in 13 per cent of the rats.

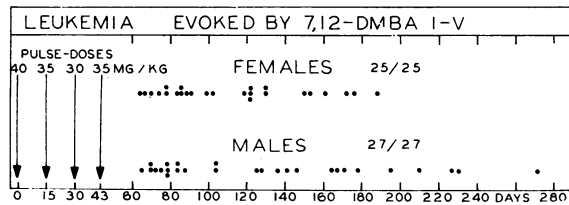


FIG. 1.—At age 27 days (day 0), male and female rats were injected with the first of four pulse doses of 7,12-DMBA. Every animal developed leukemia and was sacrificed in an advanced stage of the disease. The day of necropsy is shown.

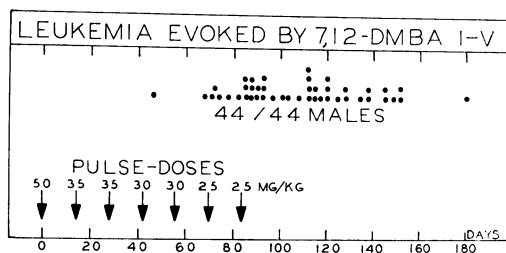


FIG. 2.—At age 27 days (day 0), male rats were injected with the first of seven pulse doses of 7,12-DMBA. Every animal developed leukemia; the day of necropsy is shown.

Types of induced leukemia: One hundred consecutive leukemic rats were classified according to distinguishing anatomical characteristics of the disease (Table 2).

(1) *Diffuse hepatic type:* Most of the leukemias (80%) were in this category, whose outstanding characteristic was the granular and fragile liver, dark red in color, which attained huge size—often more than 10 per cent of the body weight. The spleen was big (>1 gm) in about one half of the cases, but thymus and lymph nodes were not enlarged. The peripheral blood picture was in the range of that of normal animals until a late stage and then leucocytosis was slight or moderate, not exceeding 55,000/mm³. In the blood very large atypical mononuclear cells (diameter 12–20 μ) were found. Erythroblasts and normoblasts were abundant in the peripheral blood.

On histologic examination, large immature tumor cells were found increasingly to invade hepatic sinusoids until the sinusoidal endothelium was completely replaced by them. The large oval nuclei of tumor cells were strongly basophilic. In some cases there was erythroblastic differentiation in the liver. In spleen there was leukemic invasion of the red pulp with lymph follicles preserved until late in the disease. Leukemic infiltration was found constantly in lymph nodes, bone marrow, and middle layer of adrenal.

Presence of spleen or thymus is not obligatory for development of leukemia of diffuse hepatic type. The spleen and thymus of 17 female rats were removed at age 45 days; the animals were given pulse doses of 7,12-DMBA, 30 mg, on four

TABLE 2
ANATOMICAL TYPES OF LEUKEMIA EVOKED BY MULTIPLE DOSES OF 7,12-DMBA

Type of leukemia	No. rats	Leucocytes* $\times 10^3/\text{mm}^3$	Liver, % of body weight	Spleen, gm	Thymus, gm
(1) Diffuse hepatic	80	22.8 \pm 11 (6.5–54.3)	9.1 \pm 3.5 (4–18)	0.85 \pm 0.7 (0.2–3)	0.08 \pm 0.05 (0.02–0.28)
(2) Myelogenous	9	212 (23–587)	5.2 (3.3–8.6)	1.26 (0.6–2.6)	0.15 (0.09–2)
(3) Lymphoblastic	8	110 (16–285)	4.9 (3.2–8.1)	1.80 (0.4–3)	0.14 (0.07–0.26)
(4) Thymic	3	37.8 (25–47.5)	5.5 (4.2–8)	— (2.5; 2.6; 27)	6.3 (4–9.3)
None: normal female†	20	—	3.5 \pm 0.6 (2.7–3.9)	0.52 \pm 0.1 (0.36–0.75)	0.21 (0.05–0.32)
None: normal male†	77	—	2.7 \pm 0.3 (2.2–3.4)	0.59 \pm 0.1 (0.42–0.78)	0.18 (0.08–0.34)

* Leucocytes/1 mm³ venous blood.

† Age 100–200 days.

occasions with 10-day intervals beginning at age 50 days. Eight of the animals (47%) developed leukemia of diffuse hepatic type and these were found to be in an advanced stage 29–61 days after the first injection.

(2) *Myelogenous*: Outstanding characteristics were the high leucocyte count and the typical hemogram of malignant myeloid cells containing peroxidase-positive granules. Spleen is big in most of the cases. The liver was normal in size or slightly enlarged. Two of the animals had chloroleukemia characterized by the green color of thymus, lymph nodes, bone marrow, kidney, and other organs, and associated mammary carcinoma.

On histologic examination of liver, leukemic cells were found chiefly in the periportal spaces.

(3) *Lymphoblastic*: Eight per cent of the leukemias were of this type. A characteristic case had a high leucocyte count, with many cells in the lymphocytic series in the hemogram. The spleen was large but often the liver was not big. On histologic examination lymphoblastic leukemia in liver was predominantly localized in periportal spaces.

(4) *Thymic*: Three per cent of the leukemias were characterized by a huge thymus, enlarged liver, large fused lymph nodes, and a big spleen. This type of leukemia became manifest because of dyspnea and palpable lymph nodes in axillary and inguinal regions. Mononuclear cells, diameter $11 \pm 2.8 \mu$, predominated in the hemogram.

On histologic examination of liver, infiltration of stem cells was present in periportal vascular spaces.

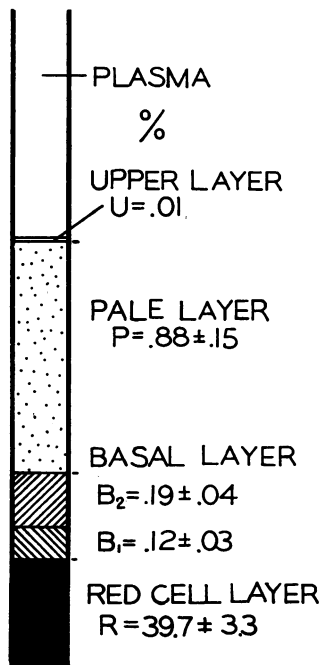


FIG. 3.—The layers of the buffy coat of blood of normal rats in the microhematocrit; the values are given in percentage.

Recognition of leukemia: Observation of the curve of body weight is helpful in recognition of leukemia in the rat. Leukemia is one of the diseases of the rat which is associated with loss of weight. A plateau in the curve of body weight followed by a progressive decline occurred in every leukemic rat; when leukemia was in an advanced stage, the decrease in weight was of the order of 2–10 gm daily.

Changes in the hemogram of venous blood appeared in relatively late stages of leukosis. Abnormalities in the number and in the differential count of leucocytes were usually preceded by anemia which became progressively severe. Icteric plasma was a sign of advanced leukemia.

Buffy coat: In the microhematocrit, the buffy coat of rat blood consists of three or four distinct layers; the mean thickness of the strata is given in Figure 3.

During centrifugation blood cells arrange according to their density, with the heaviest cells lowermost.^{13, 14} In rat blood two clearly distinct layers, which we designate B_1 and B_2 , are located above the stratum of erythrocytes; above these is a pale area, P ; inconstantly in normal blood a thin layer, U , lies above P layer at the top of the buffy coat.

In buffy coat the lower basal layer, B_1 , consists of granulocytes. The second basal layer, B_2 , is composed of mononuclear cells, chiefly lymphocytes. The third layer, P , the thickest stratum of the buffy coat, is pale yellow, finely granular, and cloudy; it consists of platelets chiefly. The top layer, U , consists of cell "ghosts" and cell fragments, but it is not present in every normal blood.

An important sign of leukemia is decrease in thickness of P layer; in many cases this stratum disappears. In addition there is an increased width of U layer, presumably associated with increased cellular destruction. In myelogenous leukemia, B_1 layer was increased in thickness; in all other types of leukemia B_2 layer was abnormally thick.

Exteriorized spleen: The translocation of spleen to a subcutaneous position facilitates palpation of this organ and the detection of nodules. Enlargement of spleen to 1+ gm is readily detected (Fig. 4) in the subcutaneous spleen.

Hepatic biopsy: A rapid method of detection of leukemia is by biopsy of liver. Histologic evidence, presumptive of leukosis, consisted of clumps of large dense basophilic cells, with huge nucleoli, located in the sinusoids or in the portal triangles.

Four pulse doses of 7,12-DMBA, 35 mg/kg, were given at 10-day intervals to 66 male rats starting at age 27 days (day 0), and hepatic biopsy was performed subsequently. On day 9, in 56 per cent of the rats, there were small focal clumps of large atypical leucocytes in the liver, whereas on day 19 changes of this sort were found in only 11 per cent of the rats. But two of the rats in this series had advanced leukemia on day 29, whereas 83 per cent of the animals had leukemia in the terminal stages on or before day 85. It would appear that in many rats the early histologic changes in liver, presumably leukosis, progressed steadily with the passage of time into advanced leukemia. But in other animals there was disappearance of the early abnormal lesions and this may be remission of leukosis; in animals of

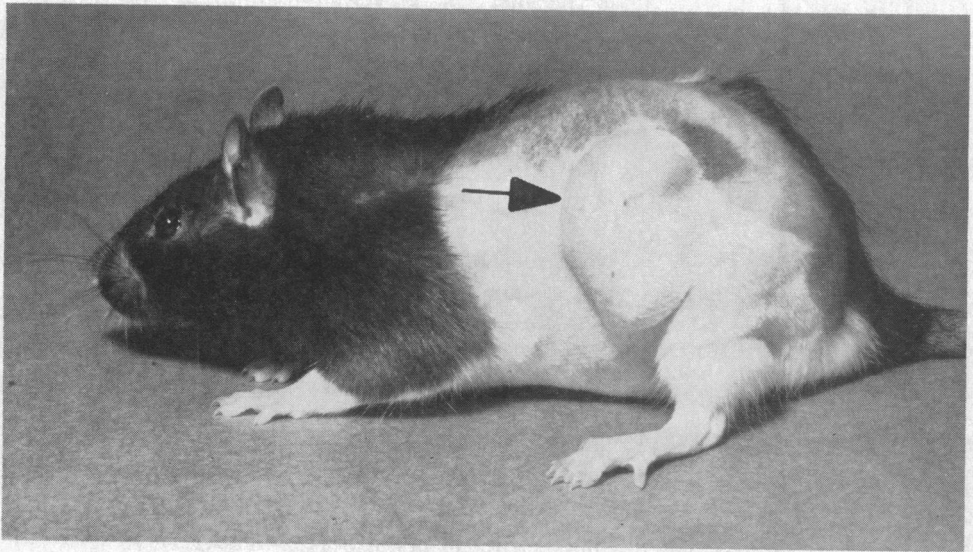


FIG. 4.—Great enlargement of spleen in lymphoblastic leukemia induced by four pulse doses of 7,12-DMBA; the leucocyte count was 13,000/mm³. Spleen had been moved to subcutaneous position 4 days before the first pulse dose.

TABLE 3
TRANSPLANTABILITY OF WHOLE BLOOD FROM LEUKEMIC DONORS

Type of leukemia	No. donors	Takes, groups	Gross Leukemia in Recipients			
			Liver	Spleen	Thymus	Lymph nodes
Diffuse hepatic	24	20/24	20/20	10/20	0	0
Lymphoblastic	2	2/2	2/2	2/2	0	0
Thymic	1	1/1	1/1	1/1	1/1	1/1

Whole blood, 0.2 ml, was transplanted i.p. into rats age 2-24 hr with five to nine recipients in a group. Gross leukemia signifies enlargement of organs with leukemia: liver >4% of body weight; spleen >1 gm; thymus >0.5 gm; lymph node >20 mg. By definition, a "take" signifies that leukemia developed in two or more members of a group of recipients.

this sort, leukemia always became evident at a later time and after additional pulse doses of 7,12-DMBA.

Transplantability of blood from leukemic rats: Whole blood, 0.2 ml, was injected i.p. into groups of rats age 24 hr or less; leukemia developed in 85 per cent of the groups (Table 3), and the type of leukemia present in the donor was reproduced in the recipients. When leukemia was successfully transmitted in this way, the disease was far advanced in 13-38 days.

The liver attained great size in newborn rats inoculated with whole blood from donors with leukemia of diffuse hepatic type, sometimes as great as 25 per cent of the weight of the body. The spleen was enlarged in one half of the animals, whereas lymph nodes and thymus were not big. Leukemic nodules were found in omentum in 55 per cent of the groups, but lesions of this sort were not associated with transplantation of blood from leukemias other than the diffuse hepatic type.

Transplantation of whole blood from leukemia of thymic type gave rise to massive involvement of thymus, lymph nodes, and, to a lesser extent, enlargement of liver and spleen.

Leukemia did not develop in 5 months in newborn rats injected i.p. with filtered plasma, 0.2 ml, from leukemic rats.

Discussion.—We identified several parameters in the process of aromatic induction of leukemia in high yield in the rat. (a) Adolescent rats were more tolerant of 7,12-DMBA than older rats were. (b) The incidence of mammary cancer induced by 7,12-DMBA was not high in rats of Long-Evans strain and it was especially low when pulse doses were initiated when the rat was adolescent. (c) Multiple pulse doses of 7,12-DMBA evoked leukemia in higher yield than a single injection did. (d) Maximum tolerated doses at intervals of 10-14 days resulted in higher yields of leukemia than the largest doses which could be given with safety to the animal at intervals of 3 days. With consideration of these factors, a simple method was devised which resulted in the efficient induction of leukemia: four or more i.v. injections of a lipide emulsion of 7,12-DMBA were given to male rats of Long-Evans strain at biweekly intervals beginning at age 27 days; the first dose was 50 mg/kg and subsequent doses were either 35 mg/kg or 6 mg, whichever amount was smaller. This schedule was well tolerated by the animals and leukemia resulted in every rat. The incidence of spontaneous leukemia in Long-Evans rats is very small.

The most helpful methods of recognition of leukemia in the rat were hepatic biopsy, exteriorization of spleen, close observation of the curve of body weight, and hemoglobin concentration and microhematocrit studies of venous blood.

Differential cell count of leucocytes in venous blood was most useful after other signs of leukosis were evident.

In 100 consecutive cases of leukemia in the rat, liver was enlarged (>4.1% of body weight) in 76 per cent of the rats. But when leukemia was in an advanced stage in any location, histologic evidence of the disease was always found by hepatic biopsy in the smaller as well as in the big livers. The spleen was enlarged (weight >1 gm) in 30 per cent of leukemic rats. Loss of weight always occurred in late stages of the disease.

Abnormal cells were frequently found in liver as early as day 9 after the first pulse dose of 7,12-DMBA, and these were clearly visible in hepatic biopsy. Some of the rats have developed leukemia which was in a terminal stage as early as day 29. In many rats there was disappearance of histologic signs, presumptive of leukosis, which had been present in liver early in the disease, but leukosis followed by leukemia reappeared with passage of time and after additional pulse doses of 7,12-DMBA.

Leukemia in the rat was associated with two types of primitive blood-forming cells. Each type resulted in leukemia with distinguishing anatomical characteristics which were pronounced late in the disease. The distinctive gross lesions in "thymic-type" leukemia were very large thymus and lymph nodes. The discriminant in "diffuse hepatic-type" leukemia was huge liver, whereas thymus was never involved. Diffuse hepatic type of leukemia, and only this type, was associated with extensive erythroblastosis.

Transplantation of leukemia to newborn rats with whole blood reproduced in most cases the type of leukemia which was present in the donor. It was noteworthy that enormous livers (often weighing 25% of body weight) occurred when cells from diffuse hepatic leukemia were transplanted; from this it is concluded that the hepatic sinusoids are a favorable locus for growth of this type of malignant cell.

Conclusion.—Multiple pulse doses of 7,12-dimethylbenz(a)anthracene, under stated conditions, induce leukosis in every rat; in most of the animals leukosis is followed by leukemia which is transplantable to newborn rats of the same strain.

Whereas several types of leukemia are evoked, the predominant type is a stem-cell leukemia which has a predilection for growth in liver and is usually associated with erythroblastosis. Presence of thymus and spleen is not obligatory for aromatic induction of this type of leukemia.

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² The following abbreviations are used: DNA, deoxyribonucleic acid; 7,12-DMBA, 7,12-dimethylbenz(a)anthracene; 3-MC, 3-methylcholanthrene; i.p., intraperitoneal; i.v., intravenous; LD₅₀, the dosage causing death of one half of the rats; ±, standard deviation.

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¹¹ We are indebted to Paul Schurr, The Upjohn Company, Kalamazoo, Michigan, for preparing a lipide emulsion of 7,12-DMBA.

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*POTENTIATION OF ONCOGENICITY OF ADENOVIRUS TYPE 12
GROWN IN AFRICAN GREEN MONKEY KIDNEY CELL
CULTURES PREINFECTED WITH SV40 VIRUS:
PERSISTENCE OF BOTH T ANTIGENS IN THE TUMORS AND
EVIDENCE FOR POSSIBLE HYBRIDIZATION**

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Rabson *et al.*¹ reported enhancement of the growth of adenoviruses in African green monkey kidney cell cultures (AGMK) following preinfection with SV40 virus. We confirmed his findings and corroborated them with other serotypes of adenovirus (unpublished observations). In the course of investigating the oncogenicity of adenoviruses grown in the presence of SV40, we observed marked enhancement of oncogenicity as well, particularly when adenovirus type 12 and SV40 virus were passed serially together for five or more passages in AGMK. The purpose of this paper is to describe the development of tumors and the virus-specific antigens found in them. Evidence of possible "hybridization," or genetic mixing, between the two viruses is also discussed.

Materials and Methods.—Cell cultures were obtained from Microbiological Associates, Inc. African green monkey kidney (AGMK), human embryonic kidney (HEK), as well as BSC-1 (a continuous line derived from AGMK)² were maintained on Eagle's minimum essential medium (EMEM) in Earle's balanced salt solution containing 3% agamma calf serum,³ 4 mM of glutamine, 100 units of penicillin, and 100 μ g of streptomycin per ml. Medium changes were done every 4 days. KB cells were maintained on EMEM as above except that the agamma calf serum concentration was increased to 10%. Medium was changed every 2 days in the case of normal KB cultures; virus-infected cultures were changed as required depending on the development of CPE.

Virus: Adenovirus type 12, strain Huie, was obtained from Dr. R. R. Rafajko⁴ and passed once at 34°C in KB cells inoculated with high multiplicity of infection. SV40 virus, strain #776, obtained from Dr. H. M. Meyer⁵ was passed four times in AGMK cells and twice in BSC-1 cells. It was propagated at 34°C using minimum virus doses, i.e., 1–10 ID₅₀ per 32 oz bottle culture.⁶

Virus assays were done at 37°C using HEK cell cultures as indicators for adenovirus and AGMK cell cultures for SV40 virus. In order to assay the SV40 infectious component in the virus mixture, titrations were done in the presence of adenovirus type 12 antiserum. Tenfold dilutions and two to three roller tubes per dilution were employed. The cultures were read twice weekly until there was no increase in titer (approximately 21–28 days). Titers were estimated according to Reed and Munch;⁷ they are given as reciprocals adjusted to 1.0-ml volumes.

The nonvirion T or neoantigen preparations were produced as described earlier.^{8–13} Adenovirus T antigens were produced in KB cell monolayers inoculated with virus multiplicities of 10 or greater and harvested at the earliest appearance of CPE or 72 hr after inoculation. The cells were scraped off the glass surface, centrifuged gently for 10 min at 1000 rpm, and resuspended in