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Physiological Thymidine Reutilization in Rat Bone Marrow.* (31159)

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The method of studying cell turnover in whole organs or tissues with the aid of a label for DNA requires a tracer which is metabolically available for a short time only and which is not reutilized following catabolism of DNA. This requirement must be met also when cell proliferation is examined over periods of several cycles.

Labeled thymidine, because of its specificity as DNA precursor and because of its rapid incorporation by cells, is widely used for turnover studies(1). However, the advantages of labeled thymidine as tracer may be offset by the fact that it is at least partially reused when DNA is catabolized(2-9). Since DNA as a carrier of genetic information in chromosomes is retained throughout the life of the cell under normal conditions (exclusive of possible repair processes), the reutilization of labeled thymidine can only begin after death of those cells that had incorporated the tracer during DNA synthesis.

A DNA catabolite, such as thymidine, its nucleotides, or even nucleotide chains, may be reutilized by direct intercellular exchange, or transported *via* the blood or lymph circulating between cells in different organs either in a free form or bound to cells acting as carriers. For example in the bone marrow, direct intercellular exchange of DNA catabolites may occur, since the most mature nucleated precursors of red cells extrude their nuclei in the process of becoming transformed into reticulocytes. These nuclei are at least partially phagocytized in the marrow(10), where catabolites of DNA consequently may become available for reutilization without mediation of the circulation. Whatever the mechanism of reutilization may be, the passage of DNA catabolites from one cell to another requires a more quantitative evaluation.

While the phenomenon of reutilization of thymidine—or its nucleotides—has been established in principle, the magnitude of this reutilization pathway and the type of material reutilized has remained largely uncertain mainly because of the lack of a proper tracer as reference for measuring true DNA renewal with exclusion of reutilization. This problem may now find a solution by using as a reference tracer the thymidine analogue labeled 5-iodo-2′-deoxyuridine (IDU), in parallel with labeled thymidine. IDU labeled with I^{131} has been used(11,12) to specifically label DNA in cells, and it was recognized that data on DNA renewal obtained with I^{131}-DU were not in agreement with results obtained with tritiated thymidine, the discrepancy being due to poor reutilization of the former in relation to the latter(2,7,9,13). The first results using IDU as reference tracer in bone marrow cells and interpreted in terms of thymidine reutilization were published previously(7,14). They are now extended and further discussed. It appears that, in rat bone marrow, 35% of the liberated thymidine, or its nucleotides, is physiologically re-

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utilized. Moreover, no isotope effect of tritium on the enzymatic reactions in the reutilization pathway was observed.

Methods. Female Sprague-Dawley rats, free of disease, were used for all experiments. They were between 3-4 months old and weighed approximately 200 g. Prior to and during the experiments the nutrition and cage condition was not altered except for changing the number of rats per cage.

The following labeled DNA precursors were used: H^3-thymidine, specific activity 1.9 C/mM (from Schwarz BioResearch, lot no. 2309); 2-C^{14}-thymidine, specific activity 30 mC/mM (from New England Nuclear Corp., lot no. 175-87a-23); and 5-I^{131}-2′-deoxyuridine, which was kindly prepared by Dr. Commerford according to the method of Prusoff (12) to a high specific activity of more than 30 C/mM. These precursors were injected into the tail vein in the following doses per g body weight: H^3-thymidine (H^3-Tdr) 0.1 μC; C^{14}-thymidine (C^{14}-Tdr) 0.01 μC; I^{131}-deoxyuridine (I^{131}-DU) 0.18 μC.

At various times after injection, as indicated under *Results,* the animals were killed with ether, one for each experimental point. Immediately after death the hind legs were cut off, and the bones were cleaned of soft tissue, split at both epiphyses, and the marrow was blown out of the bones into buffered saline. After the cells were gently dispersed into a single cell suspension and after sedimentation of the fibrinous material, aliquots were taken for triplicate cell counts in a Coulter counter properly calibrated. A precise volume of the cell suspension was then transferred to new tubes for further work up. The suspensions were always kept in an ice bath.

In side experiments a modified Schmidt-Thannhauser method(15) was used for separating acid soluble nucleotides, and RNA from DNA. The specific binding of H^3-Tdr to DNA was confirmed, and also the radioactivity attached to the residual "protein fraction" after DNA hydrolysis in perchloric acid could be ascribed to non-extracted DNA on the basis of ultra-violet light spectral analysis with correction for protein contamination(15). The acid soluble nucleotides and the saline wash (non-volatile) was found to contain less than 5% of the radioactivity bound to DNA later than one hour after precursor injection. It was 1.66% at day 2. Consequently the following techniques were used:

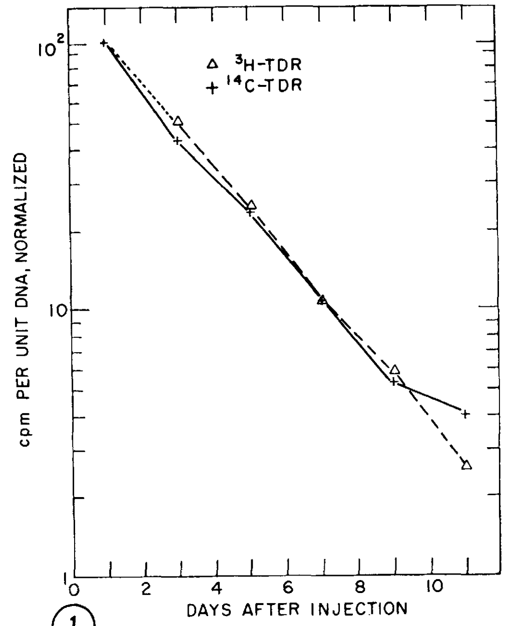
In the experiments with labeled thymidine the cells were centrifuged from the saline suspension and stored in cold 95% ethanol prior to digestion of the sediment with Hyamine, 2 ml for 10^8 cells. Fractions of the hydrolysates were counted by the liquid scintillation technique(16) in a Packard TriCarb instrument. Quenching was corrected for by using the appropriate internal standard.

In one set of IDU experiments the acid soluble fraction was extracted with cold 2% perchloric acid, and the residue was then suspended in cold ethanol prior to measuring the I^{131} directly in a conventional crystal well counter. The volumes were kept equal for all preparations. In a second IDU experiment the bone marrow was not isolated, but 2 femurs and 2 tibiae, carefully cleaned of soft tissue, were directly measured in the crystal well counter. All data are expressed in counts per minute per unit number of cells, or per 2 femurs and 2 tibiae.

The experimental error of tracer injection and nucleic acid extraction (modified Schmidt-Thannhauser method(15)) was determined 4 times on groups of 5 rats, using H^3-cytidine as tracer. For the RNA specific activity the standard deviation was 17.8%, and the standard deviation of the mean was 7.9%; the corresponding values for the DNA specific activity were 15.9% and 7.11%, respectively. These values are expectedly lower for the present experiments, because of the simplifition of techniques.

Results. Fig. 1 lists the data obtained with thymidine labeled with ^3H or ^{14}C. In both experiments the radioactivity per unit of cells regresses exponentially and in parallel with time after injection, observed from day 1 on. The half time of both regressions is equally about 2 days (46 hours), equivalent to a rate of regression of 0.015 per hour (ln 2/46).

Fig. 2 gives the regression of I^{131} activity for the total bones (2 femurs and 2 tibiae), for the the acid insoluble fraction of the



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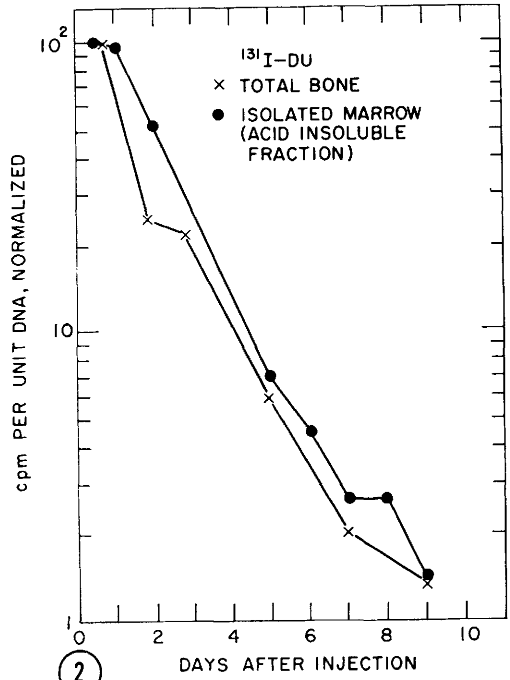

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FIG. 1. Regression of radioactivity bound to the acid insoluble fraction (DNA) of 10^6 nucleated bone marrow cells, normalized to the value at day 1, after injection of H^3-thymidine (0.1 μC/g body weight) and of C^{14}-thymidine (0.01 μC/g body weight). For the curve representing H^3 the value of day 1 was obtained by extrapolation. The standard error of injection of tracer and nucleic acid extraction was below 8% (see text, *Methods*). The counting error remained below 4.4% except: C^{14} day 9 = 9%; H^3 day 11 = 10%; day 11 = 12%.

FIG. 2. Regression of I^{131} bound to the acid insoluble fraction of 10^6 nucleated bone marrow cells, or bound to the total bones (2 femurs and 2 tibiae), normalized to the starting value, corrected for decay. The counting error of the bones remained below 3%. The counting error of the acid insoluble fraction remained below 2.5% except: day 5 = 8.5%; day 6 = 9.0%; day 7 = 11%; day 8 = 12%; day 9 = 14%.

marrow cells from these bones. Here, too, the regression proceeds exponentially from day 1 to day 9. Both curves are parallel and have half times of 1.2 to 1.3 days (30 hours), corresponding to a regression rate per hour of 0.023 (ln 2/30).

Fig. 3 shows the data of Fig. 1 and 2 combined, and the regression curves are normalized for their exponential part, drawn freehand to best fit the individual values. The discrepancy between the measurements is obvious.

Discussion. The data presented here agree with previous reports that measurements on DNA renewal in a heterogeneous cell population yield exponential regression curves after a plateau of about 24 hours(7,17). But it is clear also that the measurements are influenced by the type of labeled precursor used.

Thymidinea as tracer of DNA renewal. The parallel regression of the DNA specific activities, after labeling with C^{14}-thymidine and H^3-thymidine, indicates that both isotopes are equivalent and comparable tracers of the DNA and its catabolites in the reutilization pathway. Since the metabolic stability of tritium on thymidine is not questioned, except in the course of thymidine degradation, the constant isotope ratio in the reutilization pathway should indicate that the enzymatic reactions participating in DNA catabolism and synthesis do not discriminate against thymidine labeled with tritium. Thus a retarded reaction at one step may be compensated by an accelerated reaction at another,

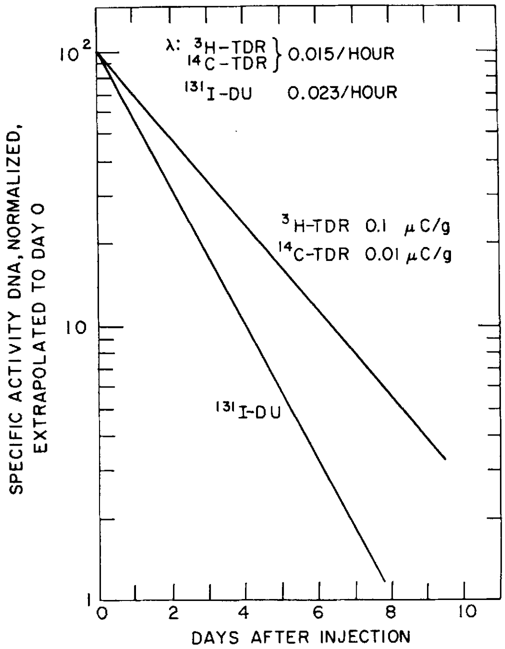

FIG. 3. Normalization of the exponential part of the regression curves in Fig. 1 and 2, extrapolated to day 0. The regression coefficient (lambda = 0.693/half time) is 0.015/hour for H^3 and C^{14}; 0.023/hour for I^{131}.

which seems unlikely. Tritium isotope effects, nevertheless, have been reported in the phosphorylation of H^3-thymidine *in vitro*(18). The lack of observation of a tritium isotope effect in the reutilization pathway agrees with the equal efficiency of incorporation *in vivo* of thymidine labeled with H^3 and with C (14,19).

The specificity of thymidine as a precursor for DNA in mammalian cells needs no further comment here although a probable reutilization of its catabolites in other chemical fractions than DNA, perhaps proteins, has been indicated(20). The similarities of the regression curves for ^{3}H and ^{14}C indicate that reutilization other than in DNA, which should be quite different for the 2 isotopes, is not important.

Thymidine is catabolized mainly and rapidly in the liver(21). Studies on thymidine catabolism by liver homogenates showed a labilization of tritium from the thymine ring to extend greatly the amount expected solely from thymidine degradation(22), and in double labeling experiments H^3 was lost to a greater extent than C^{14}. The fact that the regression curves for ^{3}H and ^{14}C in the present experiments in the intact animal are identical indicates that the thymidine from DNA breakdown which is reutilized in the bone marrow is not subject to such attack by the liver, or, if thymidine is further degraded, it is decomposed totally without salvage.

Assessing the rate of regression of the DNA specific activity after injection of labeled thymidine, it must be considered whether a continuous and delayed incorporation of labeled thymidine from some form of a storage pool occurred.

Bone marrow cells incorporate thymidine into the acid soluble fraction during the phase of DNA synthesis(23), which indicates that the "pool" renews apparently only during this period. However, storage of thymidine in the acid soluble pool may occur between 2 periods of DNA synthesis, as has been observed in *Tetrahymena pyriformis*(24) and as was indicative in L-cells in culture (25). Nevertheless storage must be infinitesimal. For all mammalian cells studied thus far, the amount of thymidine, or its nucleotides, in the acid soluble fraction is very small(1). Thus in the total mouse the entire "pool" was found to be "flooded" by as little as 0.05 μM of thymidine, *i.e.*, 12 μg(13). Moreover, the amount of acid soluble tracer in the total marrow cell population, later than one hour after injection of H^3-thymidine, was less than 5% of the tracer bound to DNA. Free circulating thymidine must pass through the liver where it is catabolized rapidly(21).

The small amount of thymidine in the acid soluble fraction, its rapid renewal during DNA synthesis, and its rapid breakdown when passing through the liver justifies the conclusion that a delayed thymidine supply from a storage pool for DNA synthesis over periods of several days cannot assume proportions to explain the slower regression of DNA specific activity.

IDU as tracer of DNA renewal. IDU is metabolically comparable to thymidine in that it is incorporated into DNA(11-13). The specificity of IDU as DNA precursor is indicated by the fact that neither in the intestine nor in the liver(13) nor in the total mouse (9), after excretion of initial IDU catabolites, could radioactive iodine be found elsewhere than in DNA. Non-specific binding of iodine soon after IDU injection into mice has, however, been observed in the liver(26), which might be expected, since the liver is the main site of IDU catabolism(13). Moreover, thymidine readily competed for IDU and as little as 0.05 μM of thymidine (12 μg) injected simultaneously with IDU depressed uptake of the latter into DNA by about 50% at 3 minutes. Ten μM of thymidine (2.4 mg) per mouse totally blocked IDU incorporation.

Also by autoradiographic evaluation of bone marrow cells in rats(27), or of sarcoma cells(28) or in lens epithelium(29), IDU was observed to be incorporated only into those cells known to be able to synthesize DNA, a finding which is complementary to the parallel behavior of the regression of I^{131} measured from the total bones and from the acid insoluble fraction of the cells separated (Fig. 2).

The specificity of IDU as a DNA precursor may be partially due to rapid catabolism of IDU not incorporated. While the incorporation, similar to thymidine(30), proceeded in the mouse with a half time of 5 minutes, the rate of breakdown showed a half time of 3 minutes. The main site of IDU catabolism, again analogous to thymidine(21), appeared to be the liver, where more than ½ of the IDU injected into the portal vein was broken down with conversion of 95% to iodine(13). Indeed, the kinetics of IDU degradation in the mouse could be explained on the basis of the liver's being the sole site of catabolism.

The efficiency of IDU incorporation into DNA was found to be lower than that of thymidine(35). In the total mouse, 5-10% of the IDU injected once was retained(13); however, 40-50% of thymidine is utilized in the rat(20). Similar data have been reported from work in humans(30).

The lower efficiency of IDU incorporation into DNA was partly explained by a preferential degradation of IDU compared with thymidine(13). Indeed, IDU was observed to be split by phosphorylase more rapidly than thymidine(31). But the preferential catabolism of IDU is probably not the only basic explanation for the less efficient incorporation of IDU into DNA. Thus a differential permeability of the cell membrane for thymidine and IDU must be considered, although nucleosides are generally known to pass the cell membrane rapidly. Further, the discriminating effect may be found in the course of phosphorylation and polymerization into DNA. This may be inferred from experiments *in vitro,* which showed the phosphokinase reaction at elevated substrate concentration to be retarded even when tritium was the isotopic marker of thymidine(18). Therefore this reaction may likewise be slower with IDU.

While the stability of iodine on smaller nucleotide chains is not yet assured, the stability of iodine on DNA is obvious from the fact that after parallel labeling with I^{131}-DU and with H^3-Tdr the isotopic ratio remains constant until labeled cells reach greatest maturity and begin to die(2), or until they are purposely killed(9). Furthermore, no loss of iodine from DNA labeled with I^{131}-DU was seen in rapidly proliferating cells in culture over a period of 3 days(14), and in non-proliferating "resting" cells in the mouse(9), where the DNA label declined over a period of more than 100 days by less than 10% only. Also in autoradiograms of bone marrow cells labeled with I^{125}-DU there was no indication of loss of tracer from labeled cells, for example, during division and subsequent maturation of myelocytes to segmented neutrophiles, or during maturation of megacaryocytes (27).

The question must be asked whether perhaps toxic effects from IDU could be held responsible for increasing the rate of decline of DNA-I^{131} over that of DNA-H^3. However, the trace amount of I^{131} immediately retained in the DNA in the experiments presented here would lead to a dose accumulation in the order of 1-2 rads. Radiation effects may not be expected at this low dose level. In experiments with I^{125}-DU, where for autoradiographic evaluation 300 μc were injected per rat and where a dose of less than 6 rads was calculated to accumulate over a period of 3 days, the mitotic index did not alter at 1 hour, 1 day, and 3 days after injection(27). Hence a lasting disturbance of cell proliferation was not manifest.

It may therefore be concluded that labeled IDU is a valid and specific tracer for measuring DNA catabolism, which is, on account of its molecular structure, less efficiently or not at all reutilized in comparison with thymidine. The regression of the DNA specific activity after labeling with I^{131}-DU, as shown in Fig. 2 and 3, in all likelihood represents therefore the measurement of DNA turnover uncomplicated by reutilization of the tracer.

Other data(2,9) on changes in specific activities of DNA after labeling with I^{131}-DU and with H^3-thymidine can be explained likewise. Thus the ratio of the specific activities with both isotopes remained constant until labeled cells began to die. For example, at a time when mature intestinal cells are known to desquamate into the bowel lumen, the I^{131} declined more rapidly than H^3(2), and irradiation of the animals at a dose causing cell death led to a greater retention of H^3 than of I^{131}(9).

Magnitude of the physiological thymidine reutilization. Degradation of DNA involves first the action of nucleases which split the molecule to mono- and poly-nucleotides. The poly-nucleotides are presumably in turn also degraded to mono-nucleotides, although alternate pathways cannot be excluded. Mononucleotides may then be used for new DNA synthesis by conversion to the nucleoside triphosphates or further degraded to nucleosides. Since the phagocytic cells which are probably responsible for the catabolism of DNA in general are a mature form not involved in DNA synthesis, it appears probable that most catabolic products must leave the cell where they are formed to be used elsewhere. Nucleotides do not readily pass through cell walls, and therefore it would appear that most reutilization occurs largely at the nucleoside level. In essence, this explains the role of nucleosides in the "salvage pathway." (Perhaps the reader should be reminded that *de novo* synthesis of nucleotides does not involve nucleosides as intermediates.)

Thymidine, when injected in tracer doses so as not to affect the pool size, has been shown to be utilized to the extent of 50% in DNA synthesis(20,30). Therefore obviously thymidine entering the circulation by catabolism must be equally utilized. The overall efficiency of reutilization of the thymine moiety in DNA might be expected to approximate this value depending on how exclusive is the utilization pathway as outlined above. Loss of thymidine due to further catabolism in the phagocytic cell before entry into the circulation would of course decrease the efficiency of reutilization; whereas reutilization of thymidine, or its nucleotide, by the phagocytic cell itself or by adjacent cells in the parenchyma would increase the efficiency of utilization.

The present data do not distinguish between the various possible intracellular routes of thymidine in reutilization, but they essentially substantiate the above interpretation. The difference in the regression curves for ^{3}H and for ^{131}I is 0.008 per hour or 35% of the rate of loss of ^{131}I activity. Assuming a uniform specific activity of the DNA in the total marrow cell population at any one time and assuming that the erythrocytic precursor cells by enucleation exclusively provided the thymidine for reutilization, one immediately arrives at a reutilization of approximately 30-40% relative to IDU. If IDU itself is reutilized to the extent of 5% then the per cent reutilization of thymidine will be proportionately increased.

The serious criticism of this quantitative interpretation may be the fact that the label was administered as a single pulse so that only cells making DNA at a given time were labeled. However, in rapidly dividing tissues such as marrow and intestinal epithelium, a majority of the proliferating cells are always making DNA. As they mature these labeled cohorts of cells undergo considerable straggling(32) so that after 1 day, or approximately 2 cell generations, nearly complete randomness of distribution of labeled cells within the various phases of the cell cycle finally results(33). Consequently, later than 1 day labeled cells arrive at the most mature stage of development together with those not labeled. This also explains why loss of activity is essentially exponential instead of stepwise as should otherwise be expected.

If the figure of 35-40% reutilization is accepted, then one can further conclude that no special mechanisms for reutilization need be involved in addition to the "salvage pathway" as outlined above. Of course this does not mean that they do not exist, but that they are probably quantitatively unimportant.

Since IDU as reference tracer for the reutilization pathway is discriminated against, in comparison with thymidine, most likely as nucleoside or as mono-nucleotide, nucleotide chains as intermediates in reutilization may escape recognition. Thus one should assume that the transfer of larger DNA units between cells—if it occurs in the mammal, as it is known from microorganisms in the course of genetic transformation(34)—is not observed in the present experiments. For clarifying this situation it is necessary to examine whether the mechanism of exchange of DNA units capable of inducing genetic transformation is altered by the presence of IDU in place of thymidine, and whether iodine remains attached to free nucleotide chains in a manner it is in native DNA.

Summary. The measurement of DNA turnover in bone marrow of the rat is influenced by the type of labeled precursor chosen as tracer. After a single injection of H^3-thymidine (0.1 μC/g body weight, spec. act. 1.9 C/mM) or of C^{14}-thymidine (0.01 μC/g body weight, spec. act. 30 mC/mM), the specific activity of DNA declined from day 1 to day 9 in a simple exponential manner with a half time of approximately 2 days (46 hours). However, after injection of 5-I^{131}-deoxyuridine (0.18 μC/g body weight, spec. act. higher than 30 C/mM), the respective half time was approximately 1.2-1.3 days (30 hours). The difference in rates of regression of DNA specific activity was discussed and attributed to reutilization of thymidine and to minimal or absent reutilization of 5-iododeoxyuridine. The parallel regression of C^{14}- and of H^3-labeled DNA after single injection of respectively labeled precursor indicates that kinetic isotope effects do not interfere with the measurement of the reutilization pathway. The magnitude of the reutilization pathway is estimated to involve 35-40% of the thymidine which is released from DNA by catabolism. The data presented here confirm thymidine to be a major contributor to the reutilization pathway. No estimates can be made with regard to the additional possible participation of nucleotide chains in the intercellular transfer of DNA catabolites.

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Human Adenoviruses: Tumor Production in Hamsters by Types 12 And 18 Grown from Single Plaques.* (31160)

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Human adenoviruses reported to date to cause tumors in hamsters(1-5) were from uncloned virus seedstocks. Thus, it is possible that the tumors resulted from agents in the stocks other than the adenovirus or that the tumors resulted from the combined action of the adenovirus and a second agent. Two sets of observations support these possibilities. First, small virus-like particles have been detected by electron microscopy in stocks of human adenovirus types 2, 5, 7 and 12(6) and of simian adenovirus type 15(7). Second, Huebner *et al*(8) reported the induction by adenovirus type 7 of tumors in hamsters having the antigenic characteristics of SV40 virus. With this exception, however, only adenovirus-specific antigens have been detected in the adenovirus-induced virus-free tumors(2,3,5), a finding that suggests the tumors were induced by adenoviruses acting alone. In addition, Trentin *et al*(1) found that only human sera which neutralized the cytopathic effects in tissue culture of adenovirus type 12 also neutralized the tumor-inducing capacity of the virus.

In this study we sought further evidence on the point of whether or not adenoviruses acting alone caused tumors by inoculating hamsters with adenoviruses prepared from single plaques of types 12 and 18. The hamsters which developed tumors also developed complement-fixing serum antibodies to both adenovirus tumor antigens(3) and the cell associated T antigens or neoantigens(9-11). The induced tumors contained antigens that reacted in the complement fixation test with specific reference antisera from hamsters bearing either type 12- or type 18-induced tumors. In carrying out these studies, we found that plaque methods described for cer-

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