Sarcoma-Producing Cell Lines Derived from Clones Transformed in vitro by Benzo[a]pyrene

Abstract. *Cells derived from mixed Syrian hamster embryo cultures were treated with pyrene (control) or with benzo*[a]*pyrene. Transformed clones were obtained only with the carcinogen. Some of the transformed clones were responsible for cell lines that produced tumors when injected into hamsters. These observations provide evidence that chemical-induced oncogenesis can be studied by an in vitro model.*

Mass cultures of Syrian hamster embryo cells treated with chemical carcinogens have been found to undergo neoplastic transformation as demonstrated by the progressive growth of the transformed cells when implanted into animals, whereas control cells failed to produce tumors (*1*, *2*). Sachs and associates (*1*) have indicated that clones from cells plated prior to or after treatment with polycyclic hydrocarbon carcinogens are capable of undergoing transformation. The "transformation" of a clone was characterized by a random crisscross pattern of spindle-shaped cells, not seen in control clones.

We have confirmed and extended this observation (*3*). Positive results were obtained with both random and inbred hamsters. The frequency of transformants was related to the known potency of the compounds tested. Toxicity increased with the amount and potency of the carcinogen. The number of transformants increased with the number of cells exposed. Certain carcinogen concentrations which produced transformation resulted in a decrease of about 50 percent in cloning efficiency, which suggests that the alteration was due to induction rather than selection.

The biological significance of this observation of alteration of chemically treated clones has not heretofore been proved by tests for tumor production. Such proof is necessary if neoplastic transformation in vitro is to offer a direct approach to the study of chemical carcinogenesis. We now report the isolation of normal and chemically altered clones and the production of serially transplantable fibrosarcomatous tumors by the use of cell lines derived from the altered clones.

In the transformation assay either primary or secondary cultures 2 to 4 days old from trypsinate of embryos of golden hamsters were used. Five hundred cells grown in Dulbecco's modification of Eagle's medium with 10 percent fetal calf serum were added to each 50-mm plastic petri dish containing 6×10^4 irradiated rat cells, as well as two sterile, precut No. 1.5 Corning cover slips adjusted to cover 90 percent of the surface of the petri dishes. In a darkened room hydrocarbon solutions were prepared and added to cultures. A chemical was dissolved in acetone and added to warm complete medium to make a stock solution. The desired concentration of chemical (0.1 to 10 μg/ml) was prepared by further dilution of the stock solution with complete medium. Solutions were added 1 day after the cells had been seeded for assay; the dishes were placed in a humidified, 10 percent CO_2 environment in an incubator kept at 37°C for a total of 10 days. No medium was changed.

Subsequently, the dishes were examined under phase microscopy for the presence of nonconfluent colonies and transformed clones. The clones were classified as either normal or transformed with a crisscross pattern, and as either semidispersed or contiguous. Some altered and normal clones were marked by circling on the bottom of a petri dish. The medium was removed, the cells were washed with warm phosphate-buffered saline, and it was verified that the cover slip had not moved. The area of the cover slip corresponding to the marked area of the dish was scored with a flamed diamond-tipped pencil.

Single pieces of cover slips each containing one colony were placed in a 25-cm^2 plastic T-flask containing 6×10^4 rat feeder cells. The medium was replaced and the cover slip shifted to a new area every 2 days. After 2 to

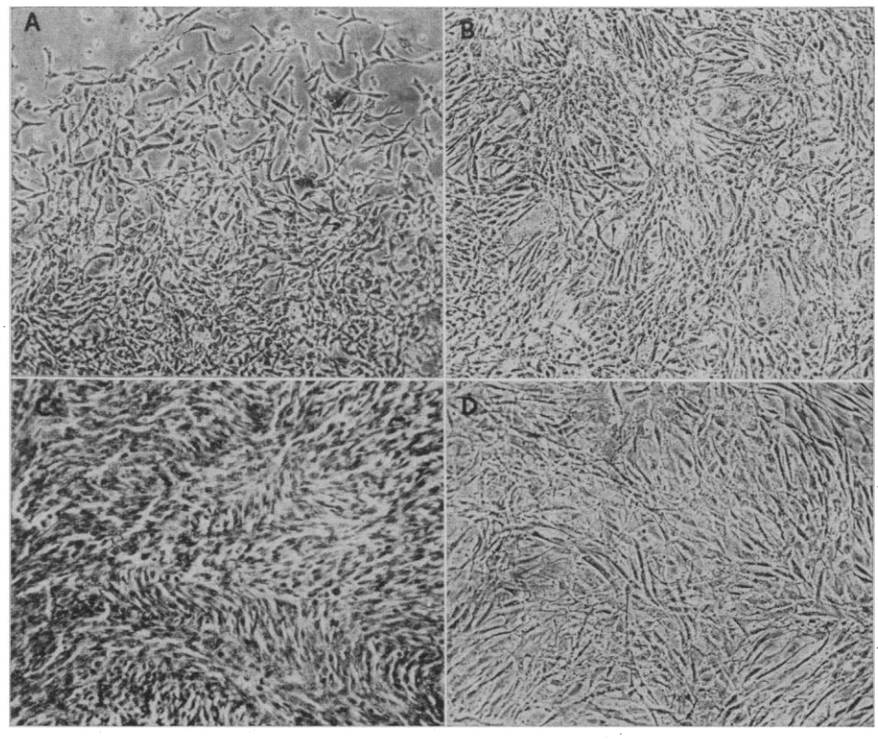

Fig. 1. (A) Colony 9 days after exposure to 10 μg of benzo[*a*]pyrene per milliliter of medium. The morphology is typical of a dense transformed colony with piling up of cells at the center and crisscross pattern at the periphery. Phase contrast (× 38). (B) Cells from 60-day-old cell line derived from above colony. Cells have large nuclei and are spindle-shaped. Some areas of growth overlap. Phase contrast (× 38). (C) Section from well-differentiated fibrosarcoma produced by the subcutaneous inoculation of 5×10^6 cells from the above 60-day-old line. Hematoxylin and eosin (× 200). (D) Typical field of a culture originated from a portion adjacent to primary tumor in (C). Cells lack orientation, and exhibit overlapping of filaments and piling up. Phase contrast (× 38).

3 weeks the flask was practically filled. The contents were trypsinized and transferred to a larger T-flask (75 cm^2) containing 1.8×10^5 rat feeder cells. A week later normal-appearing as well as transformed clones (Fig. 1A) were noted. Specific clones were again marked and, after part of the top of the flask had been removed, the clones were isolated by the cylinder technique (*4*). The cells of each clone were placed in a large T-flask without a feeder layer. Within a week sufficient transformed cells were available for injection into animals; control cells were slower growing, and 3 weeks passed before cells could be harvested for animal testing.

Thus permanent cell lines were obtained from both normal- and transformed-appearing colonies (Fig. 1B). The transformed-derived lines had many of the cell properties known as indices of neoplastic transformation, such as change in morphology and growth behavior, and detachability of cells from the surface of the flask.

To determine whether the transformed cells or the unaltered controls were neoplastic, 10^6 cells were injected intradermally or 5×10^6 cells were injected subcutaneously into weanling hamsters. Cell lines were derived from cells of fetuses of three different hamsters. In no instance did a tumor result from any of the ten control lines. Growing tumors were produced with seven out of eight transformed cell lines, five of which were derived from dense transformed clones and two of which were derived from light transformed clones. Complement fixation tests were negative for antigens of oncogenic viruses known to transform hamster cells in vitro or in vivo. Animals inoculated intradermally with transformed lines took a minimum of 4 months to develop palpable tumors which then grew rapidly and had to be retransplanted before the skin burst. Following subcutaneous inoculation of transformed cells to the dorsum, a period of 6 weeks to 3 months was required to obtain neoplasms. Although these appeared encapsulated, they grew to as much as 20 cm in diameter, and the animals became cachectic. In all cases the primary tumors were fibrosarcomas (Fig. 1C) and tumor metastasis was not observed.

The excised primary tumors were transplantable again, and cell suspensions obtained by treating minced portions of the tumors with trypsin rapidly attached to the plastic surface of the flask. Subsequently, these cells formed cultures resembling the original transformation (Fig. 1D). Particularly apparent were crisscrossing of cellular elements and the piling up of various cells.

Our observations demonstrate that cells derived from cultured fibroblasts are susceptible to neoplastic transformation of benzo[*a*]pyrene and not by pyrene. The quantitation of clonal alterations and the extent of correlation of the morphologically altered clones with tumor production require further investigation.

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References

1. Y. Berwald and L. Sachs, *J. Nat. Cancer Inst.* **35**, 641 (1965).
2. J. DiPaolo and P. Donovan, *Exp. Cell Res.* **48**, 361 (1967); T. Kuroki, M. Goto, H. Sato, *Tohoku J. Exp. Med.* **91**, 109 (1967); A. Sivak and B. L. Van Duuren, *Exp. Cell Res.* **49**, 572 (1968).
3. J. A. DiPaolo, P. Donovan, R. Nelson, *J. Nat. Cancer Inst.* **42**, 867 (1969).
4. T. T. Puck, P. I. Marcus, S. J. Cieciura, *J. Exp. Med.* **103**, 273 (1965).

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Plasmodium malariae: Transmission from Monkey to Man by Mosquito Bite

Abstract. Anopheles freeborni *mosquitoes were infected by feeding on New World monkeys,* Aotus trivirgatus, *infected with a Nigerian strain of* Plasmodium malariae. *The infection was passed to human volunteers through the bites of these mosquitoes, demonstrating the practicability of using a simian host for infection of mosquitoes with a third species of human malaria parasite and of the use of such mosquitoes to transmit the infection from monkey to man.*

The New World owl monkey, *Aotus trivirgatus,* can now be experimentally infected with three of the four human species of malaria parasites, namely, *Plasmodium vivax* (*1, 2*), *P. falciparum* (*3, 4*), and *P. malariae* (*5*). Young *et al.* (*1*) were able to infect mosquitoes with *P. vivax* which had been established in monkeys and, with the same mosquitoes, transmitted this infection back to man. We reported (*4*) the susceptibility of anopheline mosquitoes to infections with falciparum malaria in owl monkeys and the subsequent transmission of this parasite to man.

In August 1968, we isolated a strain of *P. malariae* from a Nigerian student. On 15 October 1968, blood (2 ml) from an inmate volunteer was inoculated intravenously into a splenectomized owl monkey (AO-74). On 30 December 1968, after a prepatent period of 76 days, parasites were observed in the peripheral blood of this animal. The infection was passed from monkey AO-74 by the intravenous inoculation of parasitized blood to a second splenectomized owl monkey, AO-80, where there was a prepatent period of 7 days. Prior examination indicated the absence of natural malaria infection in these animals. *Anopheles freeborni* mosquitoes fed on both these monkeys became infected and exhibited sporozoites in the salivary glands. The ease with which mosquitoes could be infected with this parasite in the owl monkey was most encouraging, since there are only a few reports of the successful experimental transmission of *P. malariae* to man (*6*).

Six volunteers were exposed to infection with *P. malariae* malaria by the bites of *A. freeborni* mosquitoes infected on monkeys AO-74 or AO-80, or both. Four volunteers were Caucasian, two were Negro. Four of the six volunteers (three Caucasians, one Negro) developed patent infections at 24, 27, 28, and 33 days after exposure. Each of these volunteers had been bitten by two or three mosquitoes with salivary glands heavily infected with sporozoites.

In order to investigate the possible relapse activity in *P. malariae* infections induced by mosquito bites under controlled conditions, all four volunteers were treated therapeutically with either quinine or chloroquine after 3 or 5 days of patent parasitemia and at least one episode of fever. By the time treatment was intiated all four patients had experienced paroxysms with temperature maximums between 39.5° and 39.9°C and parasite counts as high as 50 per cubic millimeter of blood. These volunteers are currently being observed for relapse activity of these infections.

Prior to treatment, parasitized blood was passed by intravenous inoculation from one volunteer to another of compatible blood type; patent infection ob-