

STUDIES OF DNA-INDUCED HERITABLE ALTERATION OF
MAMMALIAN CELLS*

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The chemical nature of genetic determinants was elucidated in 1944 by Avery, MacLeod, and McCarty in their classic finding of DNA-mediated transformation of pneumococcal types (1). This provided knowledge of the kinds of biochemical phenomena which are directly controlled by DNA, in bacteria. That DNA is involved in similar functions in somatic mammalian cells is not in dispute; rigorous experimental evidence however has been difficult to obtain (2). Heritable acquisition of genetic activity has resulted from the "infection" of mammalian cells by DNA isolated from oncogenic viruses (3-5), but convincing examples of transformation by mammalian DNA have been very rare indeed. Perhaps the most persuasive instance of such change is the recent report (6) that the implantation of amelanotic cells, after incubation with DNA isolated from melanin-producing embryonic cells, into white mice, induced the formation of melanin-producing nodules; unfortunately, controls with DNA from white mice or other sources were not included.

The present studies are concerned with heritable acquisition of cellular genetic properties by the exposure of cells to genetically distinct cells or to DNA isolated from them. The properties chosen were the ability to synthesize mouse gene products (antigens), and the expression of oncogenic potential. The donors were mouse Ehrlich ascites tumor cells and the recipients were non-malignant Chinese hamster cells. A preliminary account of the background and the results of our earlier experiments has appeared (7).

Materials and Methods

Cells and Reagents.—Chinese hamster (CH)¹ cells were used preferentially because their easily recognizable chromosomes ($n = 22$) greatly simplify study of their karyotypes (8, 9).

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¹ *Abbreviations used in this paper:* CH cells, Chinese hamster cells; DEAE-D, diethyl-aminoethyl-dextran; EA cells, Ehrlich ascites cells; MEM, Eagle's minimal essential medium; PBS, phosphate-buffered saline.

A line of CH embryo cells was isolated in our laboratory. Chinese hamster bone marrow cells were obtained through the courtesy of Dr. J. Biedler and HeLa cells were kindly supplied by Dr. J. Fogh, both of our Institute. Several lines of CH cells, transformed to malignancy by polycyclic hydrocarbon cancerogens or oncogenic viruses, described earlier (9) were also used. Unless otherwise noted, Eagle's minimum essential medium (MEM) with 10% fetal calf serum, penicillin, and streptomycin was used. Diethylaminoethyl-dextran (DEAE-D), mol wt about 2×10^6 , was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and thymidine methyl- ^3H (6 Ci/mole) and calf thymus DNA from Schwarz Bio Research Inc., Orangeburg, N. Y. Crystalline deoxyribonuclease (DNase) and ribonuclease (RNase) were obtained from Worthington Biochemicals, Freehold, N. J. Cultures were routinely monitored for contamination with mycoplasma (PPL0) and other microorganisms.

Co-culture Experiments.—To milk dilution bottles containing 5×10^5 Chinese hamster cells, $1-2 \times 10^5$ Ehrlich ascites (EA) cells were added. The cultures were maintained in MEM at 37°C in CO_2 incubators and subcultured twice a week by trypsinization with 0.125% trypsin-0.01% ethylenediamine tetraacetate (EDTA) after medium containing nonadhering EA cells was removed. During 4-8 wk of co-culture, randomly prepared cover slip preparations of these cells were periodically examined by light microscopy as well as by immunofluorescopy for detection of any surviving ascites cells and for the appearance of new cell types. Cells were cloned in 60 mm plastic Petri dishes to facilitate isolation and further identification and propagation of altered cells. When morphologically changed colonies were seen, they were removed with a pasteur pipet, trypsinized, and passed to fresh Petri dishes each of which contained a cover slip. This permitted removal of the cover slip for immunofluorescence studies while the cells in the remainder of the dish could be used for further propagation, recloning, and for karyotype analysis after treatment with colcemid.

To study the process of cell-to-cell interaction, co-cultures were grown in Rose-type microscope cover slip chambers (10) and examined by phase contrast optics or oblique illumination.

Radioautography.—EA or CH cells were labeled for 24 hr in culture with thymidine- ^3H (0.1 $\mu\text{Ci}/\text{ml}$), the medium removed, the cells washed twice with fresh medium, and then maintained for 2 hr in MEM containing unlabeled thymidine, 50 $\mu\text{g}/\text{ml}$. Labeled cells were then added to their unlabeled counterpart at a ratio of 5×10^4 EA cells to 10^5 hamster cells and the mixed population incubated in MEM at 37°C for 48 hr on cover slips in plastic Petri dishes. Cells were then washed with Locke-Ringer, extracted with cold 5% trichloro-acetic acid, washed twice with 75% ethanol and fixed with glacial acetic:ethanol (3:1). The slides were then dipped into 1:1 diluted NTB₃ Kodak photographic emulsion and kept in the dark for 2-3 wk. The preparations were developed and stained with Giemsa.

DNA Experiments.—About 10^5 Chinese hamster cells were seeded onto rectangular cover slips (24×40 mm) in 60 mm plastic Petri dishes, allowed to attach, and incubated 20-24 hr. Medium was then removed and replaced by 0.5 ml of a solution of EA DNA (5-40 $\mu\text{g}/\text{ml}$) in tris(hydroxymethyl)aminomethane (Tris)-buffered MEM (with 100 $\mu\text{g}/\text{ml}$ adenosine triphosphate (ATP) but no serum, penicillin, or streptomycin). The solution was placed on a still moist, less than semiconfluent cover slip, followed immediately by 0.5 ml of DEAE-D (400 $\mu\text{g}/\text{ml}$) (11) in the above "special" medium. Cultures were then kept at 37°C in a CO_2 incubator for 45 or 60 min, washed with phosphate-buffered saline (PBS), and then 5.0 ml of MEM was added and incubation was continued.

In other experiments, 5×10^5 cells were suspended in test tubes in 0.5 ml of special MEM, and 0.25 ml of DNA (40 $\mu\text{g}/\text{ml}$) was added, followed by 0.25 ml of DEAE-D (400 $\mu\text{g}/\text{ml}$), both in special MEM. On gentle swirling, a fluffy DNA-containing fiber formed in which cells were enmeshed, and incubation was continued at 37°C for 30-45 min. The fiber was then removed with a fine glass rod, and immersed in special MEM to remove excess DEAE-D. With the help of a hypodermic needle, the fiber, still containing entrapped cells, was gently spread

out on a cover slip in a Petri dish, and moistened with about 0.2 ml of special MEM. After attachment to the glass surface (about 30 min at 37°C), 5.0 ml of MEM was added for further incubation. At various time intervals, cover slips were washed with PBS and fixed with methanol for further examination by the "indirect" immunofluorescent technique (12), or trypsinized and then propagated for long term studies.

Nucleic Acids.—DNA was isolated under sterile conditions by modification of a method described by Marmur (13). Ehrlich ascites tumor cells were usually harvested 5 or 6 days after intraperitoneal inoculation into Swiss mice, washed free of blood with hypotonic PBS (diluted 1:5), and suspended in 0.15 M NaCl-0.01 M EDTA buffered to pH 7.5 with Tris. The mixture was incubated with pronase (final concentration 100 µg/ml) at 37°C for 30 min, after which 20% sodium dodecyl sulfate was added to a final concentration of 1% and the mixture kept at 55°C for 10–15 min. The very viscous fluid was diluted 1:1 with NaCl-EDTA, chilled in ice, made 1 M with respect to sodium perchlorate, and mixed for 1–2 hr on a rotator in the cold room. The preparation was then deproteinized by shaking either with 0.01 M neutral phosphate-saturated phenol or with chloroform:isoamylalcohol (24:1). After centrifugation at 7000 rpm for 10 min, the crude nucleic acids were precipitated from the supernatant with 2½ volumes of ethanol. The resultant fibrous product was dissolved in PBS by gentle overnight mixing in the cold room. The solution was treated twice with RNase (50 µg/ml) for 30 min at 37°C and again shaken with changes of phenol or chloroform:isoamyl alcohol until a protein-containing interphase was no longer discernible. The fibers obtained from the supernatant by precipitation with ethanol were washed with gradually increasing concentrations of ethanol and finally dissolved in PBS. The DNA was used as soon as possible after preparation. Small portions were frozen and kept at –20°C but never thawed more than once. In order to obtain radioactive DNA, ascites cells were labeled with thymidine-³H by intraperitoneal injection of 20 µCi of thymidine-³H into EA-bearing mice 24 hr before harvesting.

Antisera and Immunofluorescence.—Rabbit antisera were prepared by subscapular inoculation of rabbits with 10⁸ Ehrlich ascites or Chinese hamster cells a total of three times at 10-day intervals, followed by a booster after 45 days. Blood was collected 5 days later and the antisera stored at –20°C. The rabbit anti-EA sera were absorbed at room temperature and in the cold with packed cells obtained from Chinese hamster liver, spleen, and kidney homogenates. Antisera against CH cells were absorbed with normal mouse liver cells.

Fluorescein-conjugated antiserum (goat anti-rabbit IgG globulin) and rhodamin-conjugated bovine albumin, obtained from Microbiological Associates, Bethesda, Md., were reconstituted with distilled water and stored at –20°C. The fluorescein-conjugated antiglobulin was twice absorbed with packed normal mouse liver cells and diluted with an equal volume of rhodamin conjugate before use. The "indirect" fluorescent antibody technique (12) was employed to visualize the presence of antigen in methanol-fixed cells on cover slip preparations when examined in ultra-violet light with a BG-12 excitation and a #53 barrier filter, using a Zeiss Universal photomicroscope. Agfa Isopan Record film (ASA 640) was used for ultraviolet photography.

Mixed Hemagglutination Test.—For mixed agglutination tests (14), about 2×10^5 cells in 0.1 ml PBS were mixed with 0.1 ml of undiluted, rabbit anti-EA (absorbed with CH cells) or anti-CH sera (absorbed with mouse cells), previously inactivated at 56°C for 30 min. The mixture was slowly rotated at room temperature for 60 min, followed by centrifugation and three washes with excess inactivated preimmunization rabbit serum diluted 200-fold with PBS. Cells were then resuspended in 0.1 ml of the diluted normal serum and mixed with an equal volume of mouse erythrocytes (dilution, 1:50 with PBS), centrifuged, resuspended in diluted normal serum, and incubated at 37°C for 30 min. A drop of the suspension was examined microscopically with phase contrast optics.

Immunodiffusion Technique.—Ouchterlony double immunodiffusion tests (15) were carried

out with cell extracts on perforated 2% agar plates (25 × 75 mm, pH 7.0–7.2) obtained from Hyland Laboratories, Los Angeles, Calif. The plates were kept at room temperature, in moist chambers, for 24–48 hr before examination. In some instances, resolution of the precipitation lines was enhanced by staining with Amido-Schwarz 10B (Merck & Co., Rahway, N. J.). Washed cells were extracted with distilled water after freezing and thawing. The extracts were lyophilized and the residues made up in PBS at protein concentrations of 10–30 mg/ml.

Tumorigenicity.—The ability of treated cells to grow as tumors (9) was tested by injecting suspension containing 10^6 cells into the left cheek pouch of female Syrian hamsters 18–20 days old; control cells were injected into the right pouch. The animals were conditioned by injection of 0.1 ml cortisone acetate (2.5 mg) twice a week for the first 2 wk and once a week thereafter. Animals were examined over a period of 2 months for the appearance of tumors.

RESULTS

Interactions between Ehrlich Ascites Tumor and Chinese Hamster Cells.—As soon as several hours after EA and CH cells were co-cultured in Rose-type cover slip chambers, adhesions and bridges between some of them could be observed (Fig. 1). Occasionally, it appeared as though these processes were extensions of EA cells, some of which had either penetrated the nucleus of the CH cells with which they were associated, or were simply attached near this region of the cell (Fig. 1 *a, b, c*). This interaction frequently led to an elongation of the otherwise spherical EA cells, but these still retained a yellowish color which served to distinguish them from the rather grayish, flat CH cells when observed under phase contrast optics in the living state. When fixed, the EA cells appeared darker than CH cells when stained with Giemsa (Fig. 1 *d*). To confirm the cellular origin of these processes, and the possible consequences of this type of cell-to-cell interaction, the cultures were examined by immunofluorescopy.

Ehrlich ascites cells incubated with anti-EA serum, which had been extensively absorbed with Chinese hamster cells, and appropriately stained with fluorescein-conjugated anti-rabbit IgG (see Materials and Methods) showed a characteristic bright green fluorescence in ultraviolet light, as expected. This immunofluorescence reaction, specific for mouse antigens, was not given by CH cells either before or after malignant transformation by various oncogenic agents (9). When the technique was applied to co-cultures of EA and CH cells, it was evident that the intercellular connections contained mouse antigen and had therefore emanated from the mouse tumor cells (Fig. 2 *a, b*). When mixed cultures were treated with EA cell-absorbed rabbit anti-CH antisera, and stained as above, immunofluorescent “bridges” from CH cells were not observed.

Radioautography.—To study the possible intercellular passage of nucleic acid between the cells (see reference 10) in the mixed cultures, the DNA of either the EA or CH cells was labeled with ^3H -thymidine. After 24–72 hr of co-cultivation, the radioautographs revealed an occasional transfer of DNA from EA to CH cells (Fig. 3 *a, b*), but only when the cells were in intimate contact. Analogous passage of DNA from mouse leukemia L-5178 to Syrian hamster embryo

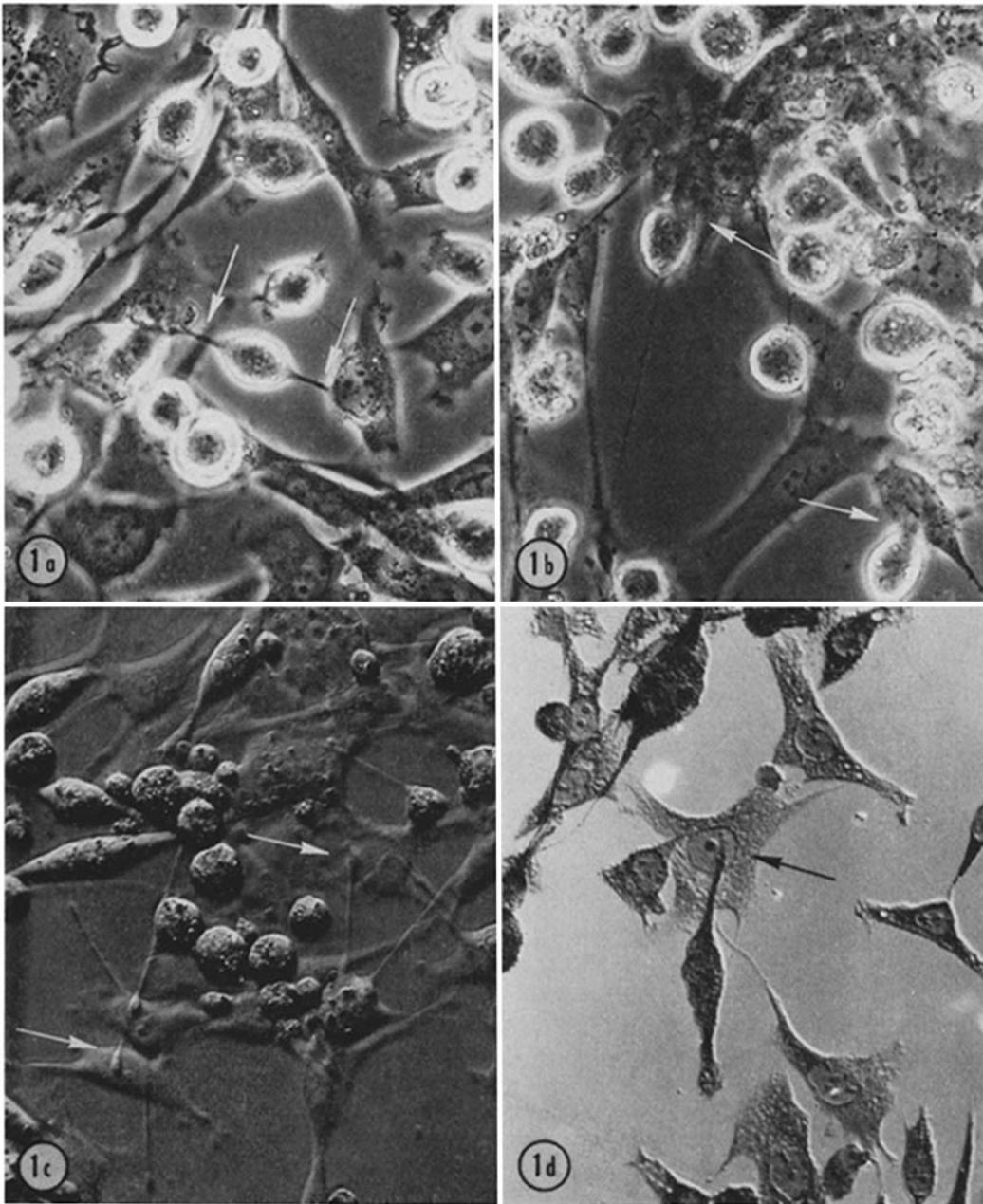


FIG. 1. Co-cultures of Chinese hamster (CH) and Ehrlich ascites tumor (EA) cells; arrows indicate bridgelike intercellular processes between the two cell types. Note that some processes are intimately associated with CH cell nuclei. (a) and (b) Photographed in the living state with phase contrast optics 5 hr after mixed cultivation. $\times 510$. (c) Photographed in the living state with oblique illumination, after 24 hr. $\times 280$. (d) Fixed specimen, Giemsa, after 24 hr; Zeiss Nomarski interference contrast optics. $\times 510$.

cells (7), and from a mouse leukemia line to mouse L cells (16) has been described in instances when these cells were cultivated together. Although the possible biological consequences of this kind of cell-to-cell interaction were not elucidated, the results below reveal the emergence of a new type of cell when EA and CH cells are grown together for extended periods.

Appearance and Isolation of New Cell Types.—After 4–8 wk of growth, the co-cultures were examined by light microscopy for the survival of ascites cells

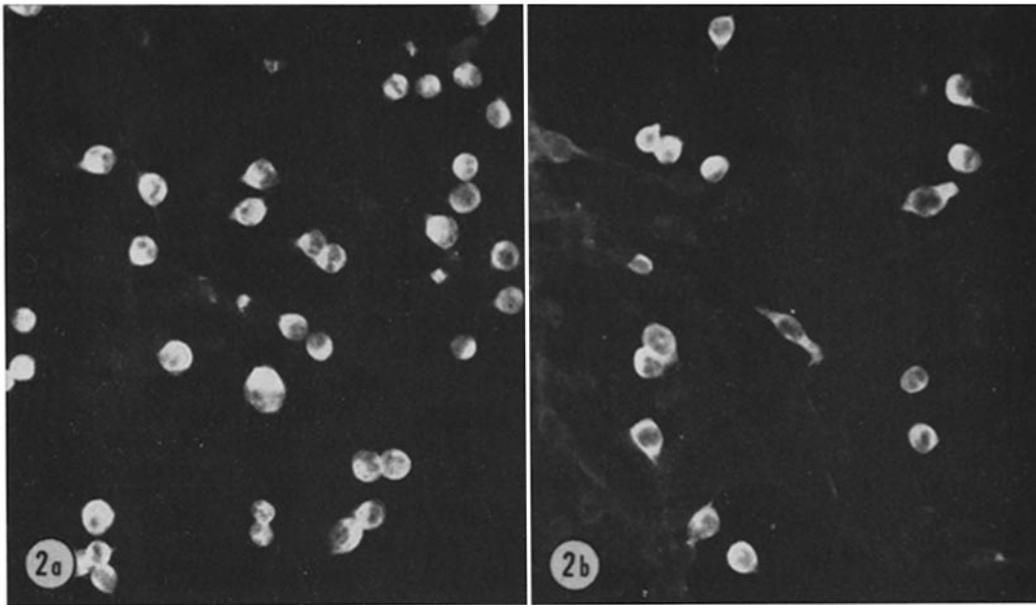


FIG. 2. Fixed 48-hr co-cultures of CH and EA cells after exposure to CH cell-absorbed anti-EA antiserum and application of indirect immunofluorescent technique; ultra-violet optics. $\times 210$. Note that the intercellular processes emanate from the brightly fluorescent EA cells making contact with nonfluorescent CH cells.

or the appearance of new cell types. Some of the spherical, suspended ascites cells, which normally do not multiply *in vitro*, can be sustained for some weeks in the presence of the CH cells which may act as a feeder layer. Unlike EA cells, the hamster cells attach to glass to form fibroblastic monolayers; accordingly, the two types are easily distinguished. The immunofluorescence technique, employing anti-EA antiserum, further assisted in the detection of surviving ascites-cells. After about 10 wk of subculturing, ascites cells were no longer detectable. Occasionally, however, a new type of cell could be observed which attached to the glass and showed the immunofluorescence reaction typical of the control preparations of ascites cells (Fig. 4 *a*). After painstaking screening

of tens of thousands of clones, about 40 were picked which appeared to consist of flatter and larger cells. From these, we succeeded in obtaining 10 clones, all the cells of which showed this EA-like immunofluorescence (Fig. 4 *b*). Since karyotype analysis revealed that these cells contained 22 or 23 Chinese hamster chromosomes (Fig. 5 *a, b*), they were not hybrids formed by fusion of ascites and hamster cells. The chromosomal pattern remained unchanged after subculturing for 1 yr; the immunofluorescence reaction was also retained. Some of

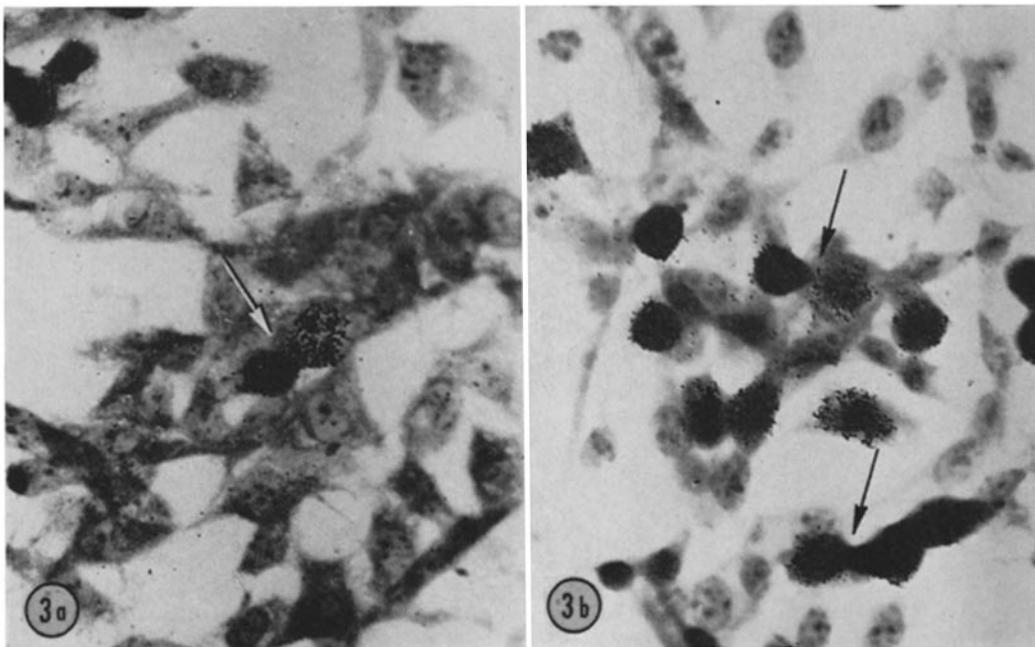


FIG. 3. Radioautographs of 48-hr co-cultures of CH and ^3H -thymidine-labeled EA cells; Giemsa. $\times 510$. Arrows indicate passage of labeled DNA from EA to CH cells. (a) Bright field. (b) Nomarski interference contrast optics.

these clones gave rise to transplantable tumors when 10^6 cells were injected into the cheek pouch of cortisone-conditioned weanling Syrian hamsters; of 38 hamsters inoculated, 6 produced transplantable tumors characterized as spindle-cell sarcomas.² None of the 125 hamsters inoculated with 10^6 Chinese hamster cells as controls developed tumors. Karyotypes of cells cultured from the tumors showed an increase in chromosome numbers from 22–23 to values of 32–38, and the persistent presence of one large submetacentric and four to seven telocentric chromosomes (Fig. 5 *c*). This pattern has remained unchanged on subculturing

² We are indebted to Dr. S. S. Sternberg for the pathology evaluation.

the tumor cells for at least 8 months; the immunofluorescence reaction was also retained. Since this submetacentric chromosome was only occasionally observed in the parent cloned cells before inoculation into animals, it may be that this cell is selected out in tumor formation.

It is tempting to regard the presence in the tumor cells of the large submeta-

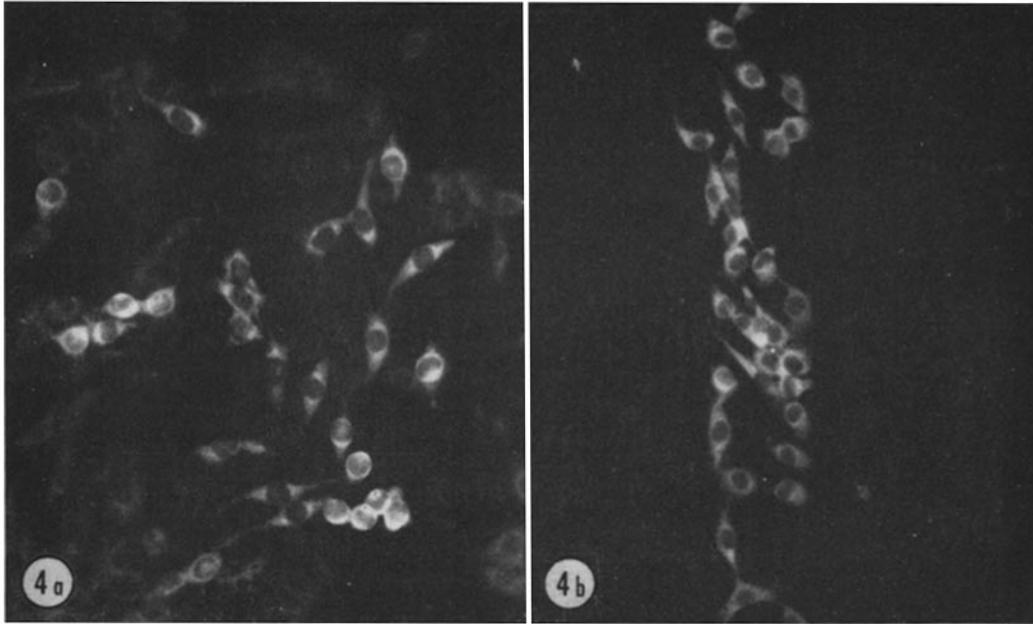


FIG. 4. Immunofluorescence reactions; ultra-violet optics. $\times 210$. (a) Co-culture of CH and EA cells after subculturing for 8 wk. New spindle-shaped cells fluoresced as if they contained murine-specific antigens when a fixed specimen was treated with CH cell-absorbed anti-EA antiserum and the indirect immunofluorescence technique was applied. (b) Cells cloned 11 wk after co-culture of CH and EA cells.

centric chromosome, seen only rarely in the cloned cells before inoculation into animals, as conferring a selective advantage for growth of these cells as tumors. This chromosome has not been seen in hundreds of karyotypes of EA cells (Fig. 5 *d*). Since the long arms of the large submetacentric are of the same size as those of the largest CH metacentrics, it may have arisen from a cleavage of the latter above the centromere position. The frequent appearance of at least one telocentric chromosome in the pretumor clones, and the presence of a few additional telocentrics in the tumor cells (see reference 9 for a similar phenomenon in CH tumor cells arising from chemical cancerigen and oncogenic

virus transformation) are examples of the kinds of karyotypic changes which may attend the cancerization process.

Mixed cell hemagglutination studies revealed a clumping of the cloned, immunofluorescent cells, derived after long-term co-culture, when these cells were

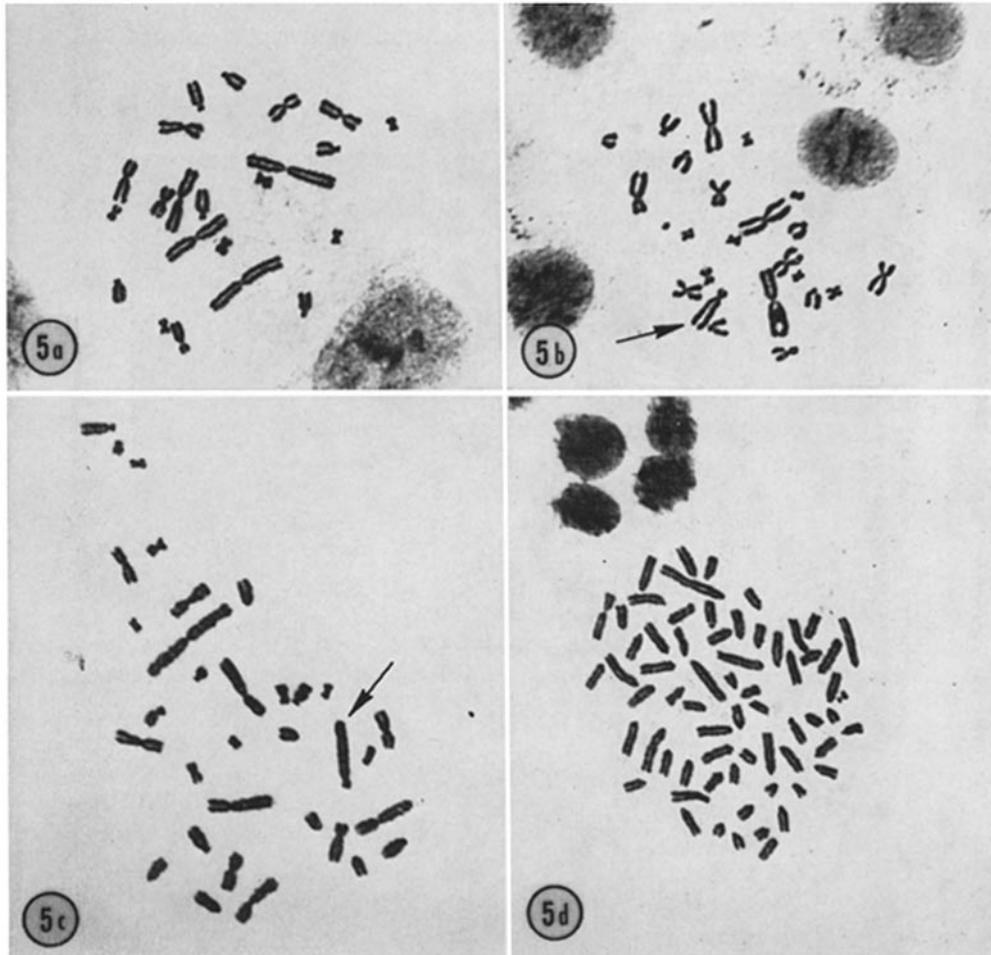


FIG. 5. Karyotypes of CH and EA cells; Giemsa. $\times 820$. (a) Parental CH cell. (b) Metaphase of new cell type derived from a clone obtained after co-culture of CH and EA cells for 11 wk. Note large submetacentric chromosome. (c) Metaphase of tumor cell obtained after inoculation of new cell type (see b) into cheek pouch of conditioned Syrian hamster. Note large submetacentric chromosome. (d) Metaphase of EA cell.

pretreated with anti-EA anti-serum, washed, and mixed with mouse erythrocytes. In contrast, control CH cells, or those transformed to malignancy by chemical cancerogens or oncogenic viruses (9), did not agglutinate when tested this way, nor did they give the immunofluorescence test. CH cells deliberately infected with PPLO were also negative. These results are consistent with the presence of murine antigens on the surface of the altered cells. Further evidence for the presence of mouse antigen in these altered hamster cells was obtained from the Ouchterlony agar double-immunodiffusion tests. Extracts of EA and altered CH cells gave a precipitin line of identity when tested with anti-EA

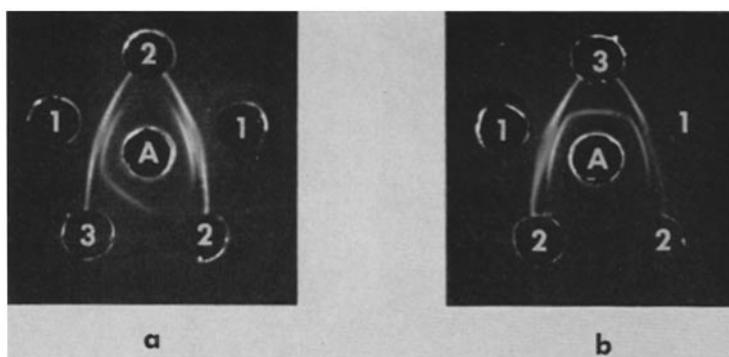


FIG. 6. Ouchterlony gel diffusion reactions; center wells (A) contain rabbit antiserum against EA cells. Wells 1 contain extract of EA cells. Wells 2 contain extract of parental CH cells. (a) Well 3 contains extract of a clone of a new type of cell (for karyotype, see Fig. 5b) obtained by co-culturing EA and CH cells for 11 wk; note precipitin line of identity with extract in Well 1. (b) Well 3 contains extract of cells (for karyotype, see Fig. 8b) cloned from a tumor derived from CH cells which had been altered after incubation with DNA isolated from EA cells; note precipitin line of identity with extract in Well 1.

antisera (Fig. 6 a). Extracts of control CH cells did not give this reaction. The lines of identity were abolished when the antiserum was absorbed with EA cells.

Interaction of Chinese Hamster Cells and DNA from Ehrlich Ascites Tumor Cells.—To study the effect of Ehrlich ascites DNA on Chinese hamster cells growing as monolayers on 24×40 mm cover slips, cells were examined 24–72 hr after incubation in the presence of DNA and DEAE-D. The seeding was such that a confluent monolayer containing about 10^6 cells formed after 72 hr of growth; the cells were trypsinized and portions reseeded to fresh cover slips or further propagated. Immunofluorescent cells were rarely observed earlier than 48 hr after incubation with EA DNA and DEAE-D; the frequency of such fluorescent cells after 72 hr was about 1 in 10,000 to 100,000, decreasing at least 10-fold when DEAE-D was omitted. This incubation interval also enabled the cells to recover from the somewhat toxic effects of the DEAE-D. When the

nucleic acid was pretreated with DNase ($1 \mu\text{g}/\text{ml}$, 30 min, 37°C), no immunofluorescent cells were observed; additional pronase and RNase however were without effect. Small foci of immunofluorescing Chinese hamster cells were observed on the monolayer cover slip preparations and extensive cloning of

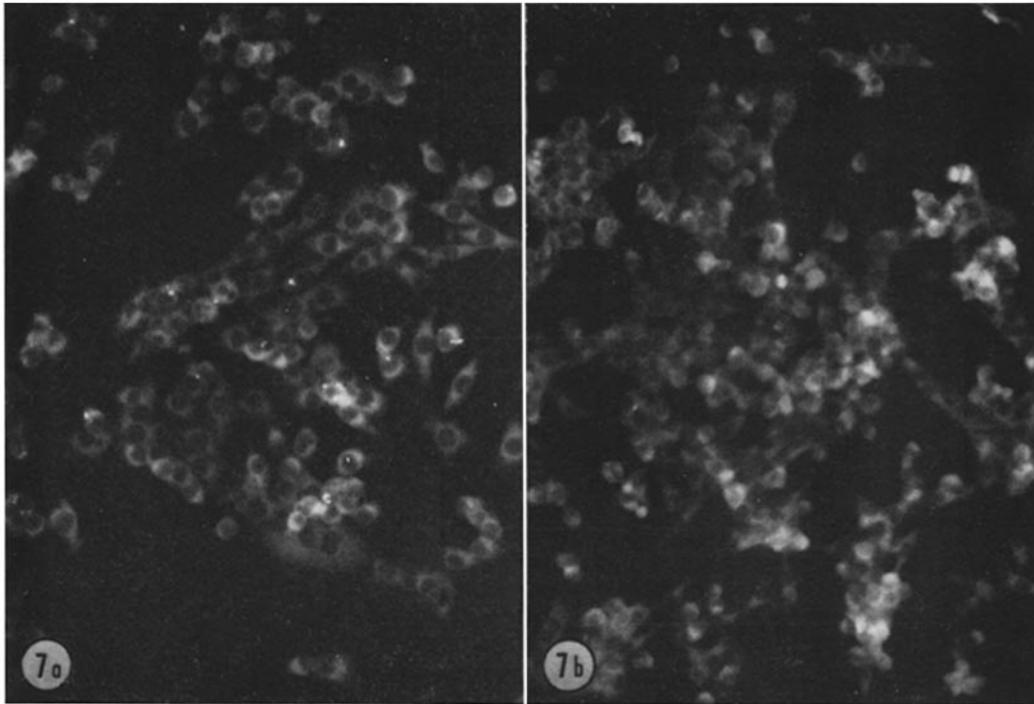


FIG. 7. Immunofluorescence reactions; ultra-violet optics. $\times 210$. (a) CH cells were treated with EA DNA for 72 hr, propagated, and immunofluorescent forms isolated by cloning. Cloned cells, above, were fixed, treated with CH cell-absorbed anti-EA antiserum, and the indirect immunofluorescence technique applied. (b) Cloned cells of tumor obtained from cells described in (a). For karyotypes see Fig. 8.

cells from replicate cover slips was carried out to help locate and isolate clones of such altered cells.

A cover slip with treated cells was cut in half; the cells on one part were propagated while the other half was examined for immunofluorescence to see whether its partner would be of interest for further study and for cloning. Since cells must be fixed before they can be examined for immunofluorescence to determine whether they have acquired the new antigenic property, the cloning procedure proved to be exceedingly tedious. However, we succeeded in isolating

17 clones, the cells of which produced the murine antigen on subsequent propagation for many months, as evidenced by the immunofluorescence reaction (Fig. 7 *a*) or the mixed hemagglutination test. Some of these gave rise to a few malignant, transplantable spindle-cell sarcomas when 10^6 cells were injected into the cheek pouch of conditioned weanling Syrian hamsters. Cloned cells from such tumors showed the immunofluorescence reaction (Fig. 7 *b*) and extracts gave immunodiffusion lines of identity (Fig. 6 *b*) with antigens of EA cells when tested with anti-EA sera. Furthermore, mixed cell hemagglutination studies also showed that the progeny from such selected clones, treated with EA-anti-

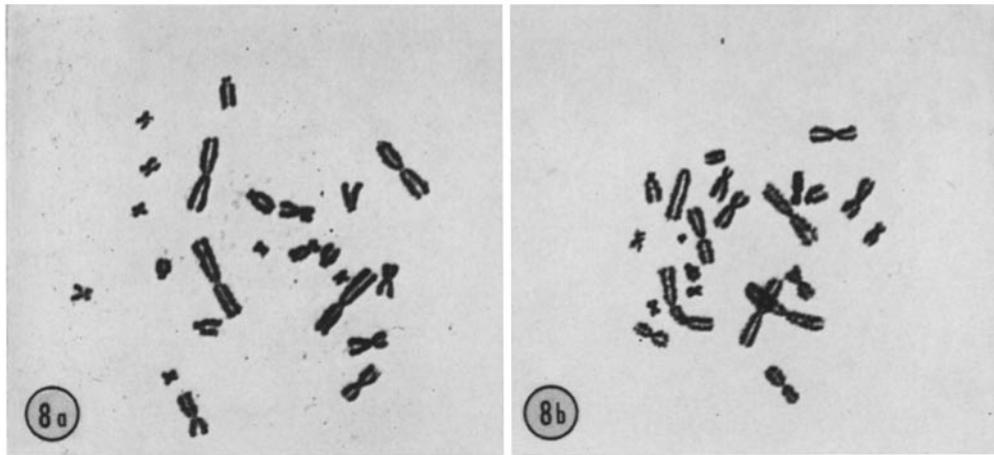


FIG. 8. Karyotypes of CH cells, following alteration by treatment with DNA isolated from EA cells; Giemsa. $\times 820$. (*a*) Metaphase of an immunofluorescent cell derived from a clone obtained after DNA treatment of CH cells. (*b*) Metaphase of immunofluorescent cloned tumor cell.

serum as above, agglutinated with murine red blood cells, thus indicating the presence and persistence of murine antigens on the cell surface.

None of the above effects were observed with Chinese hamster cells before or after they were incubated with homologous or HeLa-cell DNA, and no tumors formed when they were injected into conditioned Syrian hamsters. Extracts of tumor-producing cells, derived from CH cells by transformation with chemical carcinogens or oncogenic viruses (9), did not show murine antigens in the Ouchterlony tests employing anti-EA antisera.

In all instances, analysis of EA DNA-treated cells after cloning, showed a normal or near normal karyotype (22 or 23 chromosomes), and one of the subtelocentric chromosomes was frequently replaced by a telocentric one. The cloned cells of tumors obtained after inoculation of the above cells into hamster

cheek pouches had 22–23 chromosomes. In these instances, 1–2 of the chromosomes were telocentric. One minute and a new large submetacentric chromosome were also seen occasionally; these might have arisen as a result of a break in one of the large metacentric chromosomes (Fig. 8 *a, b*).

To learn whether the changes in CH cells following treatment with EA DNA were analogous to those observed in microbial transformation systems (17), DNA was reisolated from an immunofluorescent-positive cloned-cell population. It was again possible to induce this change, with an incidence somewhat higher than with the original ascites DNA, when DNA from altered cells was incubated with Chinese hamster cells.

Of the large series of experiments carried out with DNA, many gave negative results. Whether this can be attributed to inadequacies in technique or cloning, a special requirement for competence in the recipient cell population, or a variability in the biological activity of the isolated DNA is not known at this time. Despite improvements in understanding and technique, mammalian cell-transformation studies have often given variable results (6, 18, 19). These problems might be minimized if the efficiency of DNA-mediated change and selection of altered cells could be improved. A promising technique, still under investigation, is presented below.

Interaction of Chinese Hamster Cells with Fibers of DNA from Ehrlich Ascites Cells.—The progeny of DNA-treated cells could be more easily followed if those cells which had been affected could be preferentially removed from the cultures. Preliminary experiments in which an EA DNA fiber had been deliberately precipitated onto a CH monolayer by treatment with DEAE-D revealed an immunofluorescence in cells in the immediate vicinity of the fiber (Fig. 9 *a*) after fixation and testing in the usual way with absorbed anti-EA antisera. In a variation of this procedure, CH cells were suspended in medium containing DNA (final concentration 10–20 $\mu\text{g}/\text{ml}$); the addition of DEAE-D (final concentration 100–200 $\mu\text{g}/\text{ml}$) to the suspended cells caused the formation of fibers and the mixture was incubated at 37°C for 30–45 min. The mesh-work of fibers with many cells entrapped was lifted out of the suspension, rinsed to remove adhering toxic DEAE-D, and spread out onto cover slips by teasing it into fine fibrils (Fig. 9 *b*). Upon incubation in Petri dishes with MEM, the cells began to flatten out slowly on the glass surface and divide. There seemed to be a toxic reaction in some areas as evidenced by cell rounding and cell death. Growth was observed in other regions of the cover slip, however, especially where excessive clumping of cells with heavy fibrous material was minimized by adequate manual spreading of the fibers. Cells fixed and examined by the indirect immunofluorescence test with CH-absorbed EA antiserum 48–72 hr after incubation on cover slips frequently fluoresced brightly (Fig. 9 *c*). The test was negative when applied several hours after incubation or immediately after mixing; the murine DNA–DEAE-D fibers themselves were negative. Accordingly,

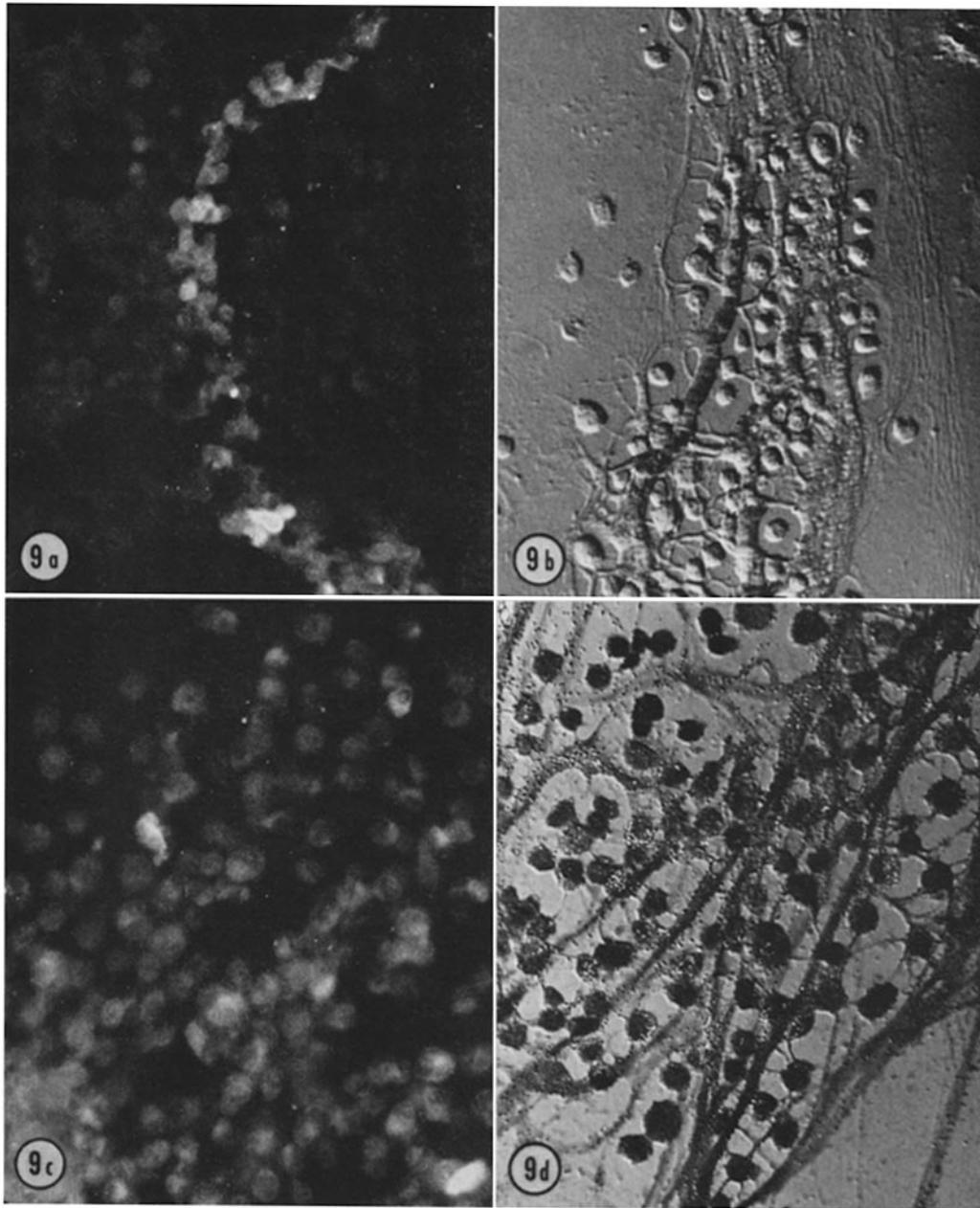


FIG. 9. Interaction of CH cells with fibers of EA DNA (see text). (a) Monolayer preparation of CH cells treated for 72 hr with EA DNA fiber; indirect immunofluorescence, ultra-violet light. $\times 210$. (b) CH cells enmeshed in EA DNA fibers, 72 hr; oblique illumination. $\times 260$. (c) Cells as in (b); indirect immunofluorescence, ultra-violet light. $\times 260$. (d) Radioautograph: interaction of CH cells with ^3H -labeled EA DNA fiber, 48 hr; Nomarski optics. $\times 510$. Note heavy labeling in many, but not all cells.

it appeared that growth of the CH cells was necessary for the positive immunofluorescence reaction which, presumably, was due to synthesis of mouse antigen.³ The reaction was not observed in CH cells treated this way with DNA isolated from HeLa cells or calf thymus.

When this procedure was carried out with EA DNA (previously labeled with ³H-thymidine), many but not all of the CH cells adhering to the radioactive fibers were heavily labeled 48 hr after incubation (Fig. 9 *d*). Further details of this procedure will be presented elsewhere.

DISCUSSION

One of the difficulties experienced by investigators of cell DNA-mediated transformation of somatic mammalian cells arises from the limited availability of markers which would demonstrate the genetic basis of DNA function as rigorously as has been accomplished in microbial transformation studies. In most of the published examples (see reviews by Ledoux [2], Olenov [20], and Glick [21]), increased resistance to antimetabolites resulted from the treatment of sensitive mammalian cells with DNA from more resistant ones (18, 19). Although a transmissible property such as resistance may involve loss of an enzyme concerned with activation of the antimetabolite, or synthesis of an inhibitor of that enzyme, direct evidence that either of these actually occurs in these examples has not yet been obtained (19). Unlike the above instances of quantitative changes, the DNA-induced appearance of melanin granules in cells of albino mice, referred to earlier (6), can be considered as an example of a qualitative transformation; in this case, the induction of synthesis of tyrosinase was inferred but not directly demonstrated.

In the present studies, we wished to see whether a mammalian cell could be directed, heritably, to synthesize gene products which had never been seen in that species of cell. This kind of change has been observed following the fusion of cells (22, 23) of two species to give heterokaryons which display gene functions of both (24, 25), a property correlated with the co-existence in the hybrid cells of chromosomes of the two species. The quantity of human antigens in a mouse-human cell hybrid appears to depend on the number of human chromosomes it contains since a decrease in the synthesis of human gene products is observed following loss of human chromosomes from the hybrid (24, 25). Total loss of human chromosomes on propagation of such interspecific hybrids, however, does not always lead to complete loss of human gene products (26); accordingly, a replicating residue of human genetic determinants was probably retained. In any case, it is evident that genes of one species can be expressed and maintained in cells of another species which ordinarily do not possess them (27).

³ CH cells enmeshed with DNA-DEAE-D fibers still incorporated ³H-thymidine in acid-insoluble form 24-48 hr after cultivation.

The mixed cultivation of mouse Ehrlich ascites tumor and Chinese hamster cells leads to the appearance of new cell forms which show morphological and cultural characteristics of CH cells (Fig. 4 *a, b*). The new cells have continued, for one year, to synthesize macromolecular cell components immunologically indistinguishable from antigens present in the mouse cells (Figs. 4, 6), and some have also acquired oncogenic potential. The karyotype (Fig. 5 *b*), which was also stable after long-term propagation, closely resembles that of the parental CH cell before (Fig. 5 *a*) or after its malignant conversion by chemical cancerogens or oncogenic viruses (9). Accordingly, the new CH cell type does not appear to have arisen as a result of cell-to-cell fusion (28). Rather, a novel interaction is involved. Bridge-like processes emanating from the EA cells (Fig. 2) develop which make contact with or penetrate the CH cells (Fig. 1); occasionally, EA DNA enters the CH cell (Fig. 3). DNA-containing intercellular bridges between cultured tumor cells (10), or nontumor cells (29–31), have been observed by others, but the biological consequence of this phenomenon has not been elucidated previously.

It is tempting to conclude that, after co-culture with EA, the heritable ability of the changed CH cells to synthesize murine-specific antigens and to grow as sarcomas arose from a functional acquisition of genetic determinants from the EA cells. Rigorous proof will require the demonstration that the altered cells are in fact progeny of a cell which had actually acquired DNA in this manner.

Although hundreds of metaphase plates were examined, it may be argued that classical karyologic analysis is not sufficient to rule out the presence of EA chromosomes (or translocated fragments thereof) in the altered CH cells as an explanation of the new properties which appear following co-culture.⁴ However, this cannot be the explanation for the acquisition of the same new heritable properties by CH cells which were incubated with isolated EA DNA. It is difficult to imagine that any chromosomes could have survived the rigorous DNA isolation and deproteinization procedures. Accordingly, we conclude that the phenomena described herein are those expected from classical DNA-mediated transformation. The ability of cells to synthesize specific antigens is certainly under gene control. Arguments that oncogenesis is also an example of altered genetic expression have been proposed (5, 7, 33–35), and the results of the present study support them. What is still ambiguous is the nature of the DNA responsible for the heritable effects obtained, and the biochemical mechanism involved. These problems are amenable to experimental study.

SUMMARY

An intercellular interaction between mouse Ehrlich ascites tumor and non-malignant Chinese hamster cells occurred when these were co-cultured. That

⁴ Application of the new *in situ* ³H-DNA-cell hybridization technique (32) should be helpful in this regard.

the intercellular processes which formed had emanated from the EA cells was revealed by immunofluorescence using anti-EA antiserum, and by direct microscopic examination. A passage of DNA from the EA to the CH cells was also observed. On long-term co-culture, new cell forms arose which were isolated, cloned, and propagated. They showed a CH karyotype and had acquired oncogenic potential and the ability to synthesize murine-specific antigens. These same heritable properties were also acquired by CH cells following their exposure to DNA isolated from EA cells.

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BIBLIOGRAPHY

1. Avery, O. T., C. M. MacLeod, and M. McCarty. 1944. Studies of the chemical nature of the substance inducing transformation of pneumococcal types. *J. Exp. Med.* **79**:137.
2. Ledoux, L. 1965. Uptake of DNA by living cells. *Progr. Nucl. Acid Res. Mol. Biol.* **4**:231.
3. Di Mayorca, G. P., B. E. Eddy, S. E. Stewart, W. S. Hunter, C. Friend, and A. Bendich. 1959. Isolation of infectious deoxyribonucleic acid from SE polyoma-infected tissue cultures. *Proc. Nat. Acad. Sci. U.S.A.* **45**:1805.
4. Habel, K. 1968. The biology of viral carcinogenesis. *Cancer Res.* **28**:1825.
5. Dulbecco, R. 1969. Cell transformation by viruses. *Science (Washington)*. **166**:962.
6. Ottolenghi-Nightingale, E. 1969. Induction of melanin synthesis in albino mouse skin by DNA from pigmented mice. *Proc. Nat. Acad. Sci. U.S.A.* **64**:184.
7. Bendich, A., E. Borenfreund, Y. Honda, and M. Steinglass. 1969. Cell transformation and the genesis of cancer. *Arch. Environ. Health* **19**:157.
8. Borenfreund, E., M. Krim, and A. Bendich. 1964. Chromosomal aberrations induced by hyponitrite and hydroxylamine derivatives. *J. Nat. Cancer Inst.* **32**:667.
9. Borenfreund, E., M. Krim, F. K. Sanders, S. Sternberg, and A. Bendich. 1966. Malignant conversion of cells *in vitro* by carcinogens and viruses. *Proc. Nat. Acad. Sci. U.S.A.* **56**:672.
10. Bendich, A., A. D. Vizoso, and R. G. Harris. 1967. Intercellular bridges between mammalian cells in culture. *Proc. Nat. Acad. Sci. U.S.A.* **47**:1029.
11. McCutchan, J. H., and J. S. Pagano. 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *J. Nat. Cancer Inst.* **41**:351.
12. Nairn, R. C. 1964. Immunological tracing: general considerations. In *Fluorescent protein tracing*. R. C. Nairn, editor. The Williams and Wilkins Co., Baltimore, Md. 3rd edition. 103.
13. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3**:208.
14. Kelus, A., B. W. Gurner, and R. R. A. Coombs. 1959. Blood group antigens on HeLa cells shown by mixed agglutination. *Immunology*. **2**:262.

15. Ouchterlony, O. 1949. Antigen-antibody reactions in gels. *Acta Pathol. Microbiol. Scand.* **26**:207.
16. Hill, M., and V. Spurna. 1968. Interaction of mouse leukemia cells with L cells in tissue culture. I. Passage of leukemic cell DNA into the nuclei of L cells. *Exp. Cell Res.* **50**:208.
17. Hotchkiss, R. D. 1955. The biological role of the deoxypentose nucleic acids. *In* The Nucleic Acids. E. Chargaff and J. N. Davidson, editors, Academic Press, Inc., New York, **2**:435.
18. Fox, M., B. W. Fox, and S. R. Ayad. 1969. Evidence for genetic expression of integrated DNA in lymphoma cells. *Nature (London)*. **222**:1086.
19. Roosa, R. A., and E. Bailey. 1970. DNA-mediated transformation of mammalian cells in culture. Increased transforming efficiency following sonication. *J. Cell. Physiol.* **75**:137.
20. Olenov, J. M. 1968. Transformationlike phenomena in somatic cells. *Int. Rev. Cytol.* **23**:1.
21. Glick, J. L. 1969. Genetic-like properties of exogenous DNA in mammalian cells. *In* Axenic mammalian cell Reactions. G. L. Tritsch, editor. Marcel Dekker, Inc., New York, 117.
22. Barski, G., S. Sorieul, and F. Cornefert. 1961. "Hybrid" type cells in combined cultures of two different mammalian cell strains. *J. Nat. Cancer Inst.* **26**:1269.
23. Harris, H., J. F. Watkins, C. E. Ford, and G. I. Schoeffl. 1966. Artificial heterokaryons of animal cells from different species. *J. Cell Sci.* **1**:1.
24. Weiss, M. C., and H. Green. 1967. Human-mouse hybrid cell lines containing partial complements of human chromosomes and functioning human genes. *Proc. Nat. Acad. Sci. U.S.A.* **58**:1104.
25. Matsuya, Y., H. Green, and C. Basilio. 1968. Properties and uses of human-mouse hybrid cell lines. *Nature (London)*. **220**:1199.
26. Midgeon, B. R., and C. S. Miller. 1968. Human-mouse somatic cell hybrids with single human chromosome (Group E): link with thymidine kinase activity. *Science (Washington)*. **162**:1005.
27. Ephrussi, B. 1967. Interspecific somatic hybrids. *In* Phenotypic expression, in vitro, Williams and Wilkins Co., Baltimore, Md. **2**:40.
28. Scaletta, L. J., N. B. Rushforth, and B. Ephrussi. 1967. Isolation and properties of hybrids between somatic mouse and Chinese hamster cells. *Genetics*. **57**:107.
29. Nelson-Rees, W. A., A. J. Kniazeff, and N. B. Darby, Jr. 1966. Chromatin bridges and origin of multinucleate cells in a bovine testicular cell line. *Cytogenetics*. **5**:164.
30. Lindholm, L., and S. Britton. 1967. Possible presence of DNA in intercellular bridges. *Exp. Cell Res.* **48**:660.
31. Buder, V. E., U. Karsten, W. Schälike, and T. Schramm. 1968. Mikrospectral-photometrischer Nachweis von Nukleinsäure in interzellulären Brücken und zytoplasmatischen Fortsätzen tierischer Zellkulturen. *Arch. geschwulstforsch.* **31**:332.
32. Pardue, M. L., and J. G. Gall. 1970. Chromosomal localization of mouse satellite DNA. *Science (Washington)*. **168**:1356.

33. Boveri, T. 1929. The origin of malignant tumors. Translated by Marcella Boveri. Williams and Wilkins Co., Baltimore, Md.
34. Horsfall, F. L., Jr. 1962. Some new concepts in biology. *Arch. Biochem. Biophys.* **1**(Suppl.):63.
35. Eagle, H. 1968. Growth-regulatory effects of cellular interaction in vitro, and their relevance to cancer. *In* The proliferation and spread of neoplastic cells. Williams and Wilkins Co., Baltimore, Md. 7.