United States
Environmental Protection
Agency

Health Effects Research
Laboratory
Research Triangle Park NC 27711

Research and Development

EPA-600/S1-81-046 July 1981

EPA

Project Summary

Improved Scoring of Chemical Transformation of C3H/10T1/2 Cells

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This research program was undertaken to improve the scoring of the transformation by chemical carcinogens of C3H/10T1/2 mouse embryo fibroblasts.

1) A probabilistic view of the transformed focus formation in these cells induced by methylcholanthrene (MCA) treatment has been formulated and validated. The authors define P_1 as the probability that a cell will be activated by carcinogen treatment, P_2 as the probability per cell generation that an activated cell will be transformed, and P_3 as the probability per cell generation that an activated cell will be deactivated. The equation derived is

$$\log(F/N) = \log[2p_1p_2(1-p_3)/2(1-p_3)-1] + n\log(1-p_3),$$

where F = mean no. of foci per dish; N = no. of cells at confluence; and n = no. of cell generations to confluence.

2) 5-Azacytidine induces differentiation of C3H/10T1/2 cells into both muscle cells and adipocytes. Although phorbol-ester related tumor promoters inhibit muscle cell formation, this is not affected by inhibitors of tumor promotion; moreover, other classes of tumor promoters do not inhibit this differentiation. Effects of promoters on adipocyte formation were inconsistent. Hence, this assay cannot be used to screen for tumor promoters.

3) The powerful tumor promoter, 12-O-tetradecanoyl phorbol-13-acetate (TPA), produces temporary and reversible rounding up of the cells and loosened adhesion to the substratum. Although TPA does not affect the growth rate of these cells in 10% fetal calf serum, it does stimulate their growth rate in 1% fetal calf serum.

4) A quantitative study of the "natural history" of clones derived from various morphological types of transformed foci was carried out. All tumorigenic Type III clones formed colonies in soft agarose, this growth efficiency increased with passage number, but only one Type II and no Type I clones grew in soft agarose. Type I clones often progressed to Type II, and many Type II clones progressed to Type III upon further passaging.

5) The cell-surface morphology was studied in the scanning electron microscope (SEM). Nontransformed cells had flat, smooth surfaces. Transformed cells in logarithmic growth exhibited rough surfaces with ruffles, blebs, and microvilli; however, the surfaces of transformed cells post confluence became smooth. "Mini-foci" of rough-surfaced

cells were visible within five days after MCA treatment.

6) **Preliminary experiments revealed that monoclonal antibodies common to transformed clones, which are probably against oncofetal antigens, can be prepared, and should be useful for scoring transformation.**
7) **Mouse peritoneal macrophages activated by BCG treatment selectively kill chemically transformed, but not nontransformed C3H/10T1/2 cells.**
8) **By mutagenesis of a tumorigenic transformed clone of these cells, followed by selection post-confluence at 39.5° with FUdR and extensive cloning, six mutants temperature-sensitive for transformed phenotypes were isolated and characterized.**

This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

It is now generally accepted that 70-90% of human cancers are environmentally caused. Since our environment contains over 500,000 naturally occurring and manmade chemicals that have not been tested for carcinogenicity, and since it costs about $500,000 per compound for a conventional large scale carcinogenesis test in hundreds of rats and mice kept throughout their lifespan, it would cost $250,000,000,000 to test them all conventionally. This is clearly prohibitive. Thus, the inescapable conclusion is that much quicker and less expensive methods of prescreening must be developed and validated.

The widely heralded Ames test appears to be highly promising as such a prescreen. There is an impressive correlation between the carcinogenic activies of 300 chemicals and their microsome-activated mutagenesis to tester strains of *Salmonella typhimurium*. However, the correlation is not perfect, there is yet no conclusive proof that carcinogenesis results from a somatic mutation, and (trite as is sounds) bacteria do not get cancer. Hence, it would be folly to rely solely on one prescreening system.

At the present time, several systems have been developed for obtaining quantitative data on the oncogenic transformation of cultured cells by chemical carcinogens. It seems clear that such systems, although more complicated and tedious than a bacterial mutagenesis assay, may be more relevant as prescreens for carcinogenic activities. They also provide valuable test materials for studying the cellular and molecular mechanisms of chemical carcinogenesis; such information will be of practical value in providing means to prevent human cancer.

In this study, a system was developed for studying chemical oncogenesis *in vitro* with the C3H/10T1/2 mouse embryo fibroblasts. However, before this system can be validated as a potential prescreen by testing in it a larger number of carcinogenic and noncarcinogenic chemicals, additional research must be done to perfect its quantitative application.

The perfection and validation of this system that determines and quantitates oncogenic transformation will produce the following benefits:

1) The availability of a reliable prescreen for environmental carcinogens (or mixtures thereof), which is one of the highest priorities in environmental surveillance.
2) Such a pre-screen can be widely applied to test large numbers of samples (pure compounds and mixtures) of environmental pollutants for their carcinogenic activity.
3) The determination of the oncogenic transformation of cultured animal cells is much more relevant to human carcinogenesis than measuring mutagenesis in bacteria or other biological or biochemical parameters.
4) The cost, space, and manpower requirements to test individual samples would be expected to be less than 1% of those necessary to carry out adequate tests for carcinogenesis in living rodents.
5) In addition to surveillance of general environmental samples for carcinogenic activity, this system could be used to monitor the changes in carcinogenic activities of specifically collected specimens.
6) The perfection of this pre-screen will also provide important fundamental information on the mechanisms of chemical carcinogenesis that, if intelligently applied, might lead to prevention of many types of human cancer.
7) Such a surveillance could lead to major steps in the abatement of carcinogenic pollutants.

Results and Discussion

Probabilistic Theory of Transformed Focus Formation

One of the original aims of the project was to apply to C3H/10T1/2 cells the transformation of single individual cells in dishes or microtiter plates, such as had been previously done with mouse prostate fibroblasts. It soon became evident, however, that the effects of cell density on transformation were so major as to obscure the very meaning of the concept of transformation frequency. This was reinforced by early reconstruction experiments in which focus formation was measured after mixing of known numbers of nontransformed and transformed cells. Here it became evident that the numbers of foci in the dishes did not follow Poisson distributions, and that there was considerable migration of transformed cells to form satellite foci. Moreover, we found that the use of microtiter plates for transformation experiments, even with single cells, was not suitable because of variations in the background and indistinctness of the transformed foci that precluded their classification as Type I, II, or III, as originally defined and studied. It soon became clear that the enormous amount of time-consuming labor that went into the preparation and inspection of the dishes containing single cells, as well as the enormous number of dishes that would be required to obtain statistically significant results, precluded this approach to improved quantitation and scoring of this bioassay system. These complexities led to a theoretical and experimental examination of a probabilistic theory of transformed focus formation, which should have major impact on the field.

Quantitative Study of the Properties of Cells Derived from Various Types of Foci

In this study, a rigorous investigation was made of various of the transformed phenotypes in a series of cell lines derived as defined below, in order to relate the "natural history" and evolution of these cultures to the phenomenon of tumor progression. In a variety of cell

culture systems an extremely tight correlation has been observed between the ability of a transformed cell to grow when cultured in semi-solid media (anchorage independence for growth) and its ability to grow as a tumor *in vivo*. At the inception of this study, very little was known about the relationship between C3H/10T1/2 transformation and anchorage independent growth. The acute need for cell culture correlates with *in vivo* tumorigenicity prompted the initiation of this work. The ability of cells to grow in semi-solid medium was assayed following a modification of the basic procedures of Macpherson for soft agar culture.

Agarose was found to be superior for the culturing of 58MCA Cl 16 cells, permitting the formation of large colonies with a high plating efficiency. An agarose overlay concentration of 0.25% was found to be optimal. Higher overlay concentrations reduced colony sizes. Lower agarose concentrations were too dilute and colonies were observed to grow, migrate, fragment and fuse.

Once the optimal conditions for soft agarose culture had been determined, C3H/10T1/2 Cl 8 and a number of transformed variants were assayed for anchorage independence of growth. All eight tumorigenic cell lines tested grew in soft agarose culture. In general, all transformed cell lines fell into one of two classes with respect to the size of the colonies formed in soft agarose culture. Most of the cell lines grew to form only small colonies (25-200 cells) but some cell lines grew to form very large colonies containing 1000 or more cells.

The ability of cells to grow as tumors was assayed in syngeneic C3H mice. Mice were X-irradiated (450r) 24 hours prior to the injection of cells. Cells ($2x10^6$) were then inoculated subcutaneously and the animals examined weekly for six months for the presence of tumors.

Of the five nontumorigenic cell lines tested here, three (including C3H/10T1/2) showed no, or very poor, capacity for growth in soft agarose.

The ability of a cell line to grow in soft agarose increased as a function of the number of passages it is maintained in culture, since the plating efficiency and colony size of cells grown in agarose increased with passage number. The results with DMBA Cl III are particularly noteworthy. These cells were nontumorigenic when tested at passage 7, but were tumorigenic when tested at passage 15. The acquisition of tumorigenicity in these cells appears to be accompanied by an increase in the ability to grow in soft agarose.

The fact that none of these early transformed cell lines were tumorigenic at early passages, whereas 58 MCA Cl 16 was highly tumorigenic, suggests that oncogenic transformation occurs in several steps or that the transformed cell lines are too strongly antigenic to induce tumors in C3H mice that were immunosuppressed by 450 rad X-irradiation. The feasibility of using intracranial injections or nude mice for tumorgenicity studies is now under study.

Morphology of Nontransformed and Transformed Cells in the Scanning Electron Microscope (SEM)

Subconfluent cultures of C3H/10T1/2 cells taken at various times following treatment for 24 hr with 1.0 μg/ml of MCA were examined in the SEM. The surface morphology of untreated cells is flat and featureless.

This study recently discovered that these carcinogen-treated cultures form a new transformation parameter, "mini-foci," which are seen also in cultures derived from Type II and Type III foci. Mini-foci are groups of cells growing on top of each other in a criss-cross fashion and are detected under subconfluent culture conditions. They have been detected in MCA exposed cultures as early as four and seven days following treatment. Similarly grouped cells have also been found in 7-day cultures treated with MCA (0.1 μg/ml, 24 hr) alone or MCA (0.1 μg/ml, 24 hr) plus TPA (0.1 μg/ml, four days after MCA treatment) but apparently in much lower frequency. Mini-foci have not been detected in control-cultures.

SEM studies of carcinogen treated confluent cultures proved uninteresting and did not reveal differences in cell surface morphology that could be definitely identified as similar to or characteristic of transformed (Cl 16) cells. This was true even with cells in foci-like areas identifiable by light microscopy. Because of these rather disappointing results, the author proposed to reexamine certain assumptions and start with cells from foci of transformed cells detectable by light microscopy. Sublines of cells isolated from the three different types of foci - Type I, Type II and Type III were studied. A "Type 0" subline, isolated from the "flat" areas between foci of cells in carcinogen exposed cultures, was also available. These, as well as controls (subline from acetone treated cells) were coded and processed for SEM examination. The cultures were seeded as usual (5000 cells per dish) and fixed for SEM at 4, 5 (subconfluent), 8 (confluent), and 14 days. The identity of each sample was checked only after completion of SEM examination.

SEM studies of these samples yielded encouraging results with subconfluent (4, 5 days) cultures. Cells with altered morphology and distinguishable from controls were easily seen in Type III and Type II cultures. These cultures had cells that often occurred in groups and tended to overlap each other in a criss-cross fashion. These "mini-foci" were taken to be significant in view of their similarity to the foci seen in the light microscope, and are considered to be diagnostic of transformed cells. Additionally, many cells in these early cultures showed a profusion of microvilli and blebs on their surfaces, and presented a morphology similar to Cl 16 or other oncogenic transformed cell populations. Type I cells were mostly non-overlapping and had fewer altered cells with microvilli and blebs, but their sparseness made scoring of these cultures as transformed difficult and equivocal. Type 0 cultures were similarly equivocal. Control, acetone treated cultures at similar subconfluency showed little or no detectable criss-cross cells, but contained cells with microvilli and ruffles. These cells at the relatively low (less than 400x) magnifications commonly used could not be distinguished as being different from those seen in the Type 0 and Type I cultures. Thus, distinctions between Type I, Type 0, and A (acetone) controls are equivocal and subjective. Experience suggests that quantitative analyses should be attempted in the future to make clear the distinctions, if any, among these cultures.

In other experiments, cells from Cl 16 (MCA-transformed, tumorigenic) were examined after growth on glass or plastic surfaces under confluent and subconfluent conditions. The results of this study showed easily detectable cells with transformed morphologies in Cl 16 cultures. Subconfluent cultures were best for detection of the transformed cells. Cl 16 cells in comparison to control cells appeared less tightly adherent to the growing surface (more

rounded). Cell surfaces often contained blebs and microvilli. Microvillar distribution and size often appear to be heterogeneous. Cell criss-crossing was also noted. This overall morphology was maintained even in five-day-old cultures, a time at which control cultures are well-spread, actively growing and often nearly confluent. Confluent Cl 16 cultures were much less remarkable in appearance, had many fewer cells with large blebs or microvilli, and generally were difficult to distinguish from control 10T1/2 cells. Nonetheless, closer examination showed subtle changes in cell-cell associations and cell shape.

In summary, the experiments done so far permit the conclusion that transformed cells are recognizable by scanning electron microscopy at early times (within one week of carcinogen exposure) in terms of changes in cell surface morphology, and/or cell shape and/or cell to cell association (close, tight adhesion vs. loose contact; criss-cross, piled vs. parallel oriented, contact-inhibited). Secondly, the expression of these changes is dependent upon culture conditions such as cell density. Thirdly, it was noted that treatment of C3H/10T1/2 cells with two different chemical carcinogens can result in dissimilar but transformed phenotypes.

Development of Monoclonal Antibodies to Oncofetal Antigens on Transformed Cells

For the prototype experiments, two oncogenically transformed clones were chosen that had previously been shown to exhibit the oncofetal antigen: MCA Cl 15 and DMBA Cl 2. C3H mice were immunized three times at weekly intervals with $5x10^5$ DMBA Cl 2 cells by intraperitoneal injections and were bled three days after the last immunization. The binding capacity of the antibodies to the transformed cells was determined by the binding of protein A. This protein, which is a single polypeptide isolated from the cell wall of *Staphylococcus aureus*, has an extraordinary affinity of binding to the Fc region of the immunoglobulin IgG. Thus, the assay measures the amount of protein A (labeled with ^{125}I) that binds to the Fc portion of the antibody, which in turn binds to the surface antigen of the intact cell through the Fab portion of the antibody. The transformed cells ($1x10^5$) were plated in Falcon 96 well microtiter plates and allowed to attach for 12 hours. They were then washed three times with Basal Medium Eagle's (BME) with 1% bovine serum albumin. The cells were then treated for 30 minutes at 4°C with the mouse antiserum or various dilutions thereof in BME. The cells were again washed 3 times with BME and incubated for 1 hr at room temperature with 10^5 cpm of ^{125}I-protein A (Pharmacia) radioiodinated with chloramine T (86) in phosphate buffered saline (PBS), and then washed three times with PBS. The wells of the microtiter plate were sawed out, placed in liquid scintillation vials, and the bound ^{125}I was counted. The antiserum obtained from DMBA Cl 2 immunized mice bound to those cells and to MCA Cl 15 cells to approximately the same extent and to a significantly higher extent than to the non-immune serum control at dilutions of 1:5. This similarity in extents of binding suggests that the antibody bound to a common cell-surface antigen on both transformed cells.

There was no significant binding of nontransformed C3H/10T1/2 Cl 8 cells to antisera prepared against those cells or to the antiserum prepared against the transformed DMBA Cl 2 cells. Moreover, the transformed DMBA Cl 2 cells reacted with the antiserum directed against the same cells, but not with the antiserum to the nontransformed cells. These two experiments demonstrated that the cells and assays used were sufficiently selective and sensitive to allow us to proceed to prepare monoclonal antibodies from hybridomas.

C3H mice were immunized three times once weekly with $5x10^6$ DMBA Cl 2 transformed cells. Three days after the last immunization, spleen cells were collected and fused with a mouse myeloma cell line, P3 x 63/Ag8, that lacks thymidine kinase using polyethylene glycol. After completion of the cell fusion and selection in HAT medium for two weeks, the cells were then cultured in regular medium. After the fourth week in culture, the supernatants of the hybridomas were added to target DMBA Cl 2 cells plated in microtiter wells as described previously and assayed by ^{125}I protein A binding. The wells were either counted for ^{125}I binding, or the pattern of bound ^{125}I was determined by autoradiography at -70°C. In a typical experiment, 4 out of 120 hybridoma wells contained antibodies against the transformed cells. Experiments are now in progress to prepare pure clones of the hybridomas that secrete antibodies of the desired properties.

Specific Killing of Transformed Cells by Activated Peritoneal Macrophages

The first approach to this problem involved setting up an assay for the killing effect of activated peritoneal macrophages. Since much of our work involves determination of the reproductive capacity of cells by measuring their plating efficiency, and since this assay has not been applied to macrophage killing, this method was used and compared with the $^{125}IUdR$ release assay.

The peritoneal macrophages of C3H mice were activated by injecting IP 1 ml of BCG containing $2 x 10^7$ live organisms. Nine days later, the mice were injected IP with 2 ml of thioglycollate 5 days before harvesting the macrophages. After harvesting, the cells were counted and plated in 100 mm dishes ($2x10^6$/dish) with 10 ml of Basal Medium Eagle (BME) + 10% fetal calf serum (FCS). After one hour's incubation, the medium was aspirated out; the adherent cells are practically all (90-95%) macrophages. We then plated 5000 of our test cells with 10 ml of BME + 10% FCS and incubated for 48 hr. At the end of this time all the cells were scraped from individual dishes and replated, without counting, into 10 100 mm dishes with 10 ml of medium in each. The dishes were incubated for 10 days and fixed with methanol, stained with Giemsa and scored for the development of colonies. The results demonstrate that the activated macrophages recognize the malignant DMBA Cl 2 and MCA Cl 16 cells and kill them, as shown by the decrease in number of colonies. In contrast, the nontransformed C3H/10T1/2 cells were not killed by the macrophages and the number of colonies was actually increased as compared to the dishes without macrophages. This is due to a feeder effect.

An exactly comparable experiment was then set up with the nontransformed C3H/10T1/2 Cl 8 cells, and with acetone-treated and Types 0, I, II, and III-derived cell lines at passage 9. None of these cell lines at this passage number grew significantly in soft agarose. The activated macrophages again enhanced the plating efficiency of the C3H/10T1/2 Cl 8 cells, but produced no significant effect on the reproductive capacity of the others except for a marginal killing effect on the Type III cell line.

Dr. Michael Fisher of the Frederick Cancer Center has performed on our

ells a newly developed microassay, hich is carried out in 96-well microtiter lates. Log phase target cells are preabeled for 24 hours with 0.2 μCi/ml of ^{25}IUdR washed three times, and trypsinzed and counted. Mouse peritoneal iacrophages were activated in culture ith macrophage activating factor (MAF) 78) for 24 hours. Then 10^5 viable trypan blue) macrophages (MP) are dded to each microtiter well. After 45 iin the macrophages were washed hree times to remove nonadherent ells, and 5×10^3 target cells were added o each well so that the ratio of macroihages to target cells added was 20:1. \fter one day, the cells were washed to emove nonadherent cells, and incubaion was carried on for three additional 'ays when the cells were washed three imes and incubated for one hour with):1 N NaOH, and the radioactivity of the \aOH lysate was determined in a gamma :ounter. In all experiments, controls vere run in which the target cells were ncubated with nonactivated peritoneal nacrophages. The target cells were ;ent to Dr. Fisher at the 9th passage, ind were tested by him at the 14th)assage.

In these experiments there was no ;ytolysis produced by the nonactivated nacrophages of any of the cell lines, or)y the activated macrophages on the iontransformed C3H/10T1/2 Cl 8, icetone-treated, or Type I cells. How-;ver, there was significant lysis of the 'ypes II and III cells, as well as of the)ncogenically transformed MCA Cl 16 :ells. It is not known whether the differences in results between these experiments and the ones done by)lating efficiency are due to the dif-'erence in the assay, the difference in he method of activation of the macro-)hages, or the difference in the passage iumber of the Types II and III cells. It is :lear, however, that the micro ^{125}IUdR assay is quicker, easier, requires less :ells and media, and is more accurate han the plating efficiency assay. Hence, all future experiments will be done by he micro radioactivity assay.

Conclusions

All work supported by this grant was aimed at improving the scoring, and making it more rapid and quantitative, of the C3H/10T1/2 mouse embryo fibroblast system for oncogenic transformation and its initiation and promotion. The following conclusions have been drawn:

1) A probabilistic view of transformed focus formation in these cells induced by methylcholanthrene (MCA) treatment has been formulated and validated. We define P_1 as the probability that a cell will be activated by carcinogen treatment, P_2 as the probability per cell generation that an activated cell will be transformed, and P_3 as the probability per cell generation that an activated cell will be deactivated. The equation derived is

$$\log(F/N) = \log [2p_1p_2 (1-p_3)/2(1-p_3)-1] + n\log (1-p_3),$$

where F = mean no. of foci per dish; N = no. of cells at confluence; and n = no. of cell generations to confluence.

2) 5-Azacytidine induces differentiation of C3H/10T1/2 cells into both muscle cells and adipocytes. Although phorbol-ester related tumor promoters inhibit muscle cell formation, this is not affected by inhibitors of tumor promotion; moreover, other classes of tumor promoters do not inhibit this differentiation. Effects of promoters on adipocyte formation were inconsistent. Hence, this assay cannot be used to screen for tumor promoters.
3) The powerful tumor promoter, 12-O-tetradecanoyl phorbol-13-acetate (TPA), produces temporary and reversible rounding up of the cells and loosened adhesion to the substratum. Although TPA does not affect the growth rate of these cells in 10% fetal calf serum, it does stimulate their growth rate in 1% fetal calf serum.
4) A quantitative study of the "natural history" of clones derived from various morphological types of transformed foci was carried out. All tumorigenic Type III clones formed colonies in soft agarose, this growth efficiency increased with passage number, but only one Type II and no Type I clones grew in soft agarose. Type I clones often progressed to Type II, and many Type II clones progressed to Type III upon further passaging.
5) The cell-surface morphology was studied in the scanning electron microscope (SEM). Nontransformed cells had flat, smooth surfaces. Transformed cells in logarithmic growth exhibited rough surfaces with ruffles, blebs, and microvilli; however, the surfaces of transformed cells post confluence became smooth. "Minifoci" of rough-surfaced cells were visible within five days after MCA treatment.
6) Preliminary experiments revealed that monoclonal antibodies common to transformed clones, which are probably against oncofetal antigens, can be prepared, and should be useful for scoring transformation.
7) Mouse peritoneal macrophages activated by BCG treatment selectively kill chemically transformed, but not nontransformed C3H/10T1/2 cells.
8) By mutagenesis of a tumorigenic transformed clone of these cells, followed by selection post-confluence at 39.5° with FUdR and extensive cloning, six mutants temperature-sensitive for transformed phenotypes were isolated and characterized.

Several of the initial objectives have been realized, and sufficiently promising preliminary data have been collected to make it highly likely that continuation of this research will lead to the accomplishment of all the objectives.

Recommendations

The numbers of these recommendations pertain to the numbers of the conclusions previously stated.

1) The probabilistic view of transformed focus formation of C3H/10T1/2 cells following chemical carcinogen treatment has been derived and validated. This is expected to have a major impact on the quantitation and interpretation of transformation in this transformation system that is now widely used throughout the world. This research should be continued.
2) The effects of tumor promoters on the 5-azacytidine-induced differentiation of C3H/10T1/2 cells into muscle cells and adipocytes may be useful in establishing the mechanism of action of phorbol ester-related tumor promoters. However, inconsistent results preclude this from being used as a short-term test for tumor promoters. Hence, this approach has been discontinued.
3) Since the transient rounding-up of cells following TPA treatment does not occur on repeated treatments, which are required for promotion

of transformation, this effect is unlikely to be involved in tumor promotion. The growth-stimulating effect of TPA in 1% fetal calf serum, seems unlikely to be involved in the mechanism of promotion because promotion experiments are carried out in 10% fetal calf serum in which no growth stimulation was observed. Hence, these approaches are being discontinued.

4) The study of the "natural history" of transformed foci has been complicated, and further studies are required to fully assess the significance of the various transformed phenotypes.
5) The appearance of "mini-foci," detectable in the scanning electron microscope (SEM) only a few days after chemical carcinogen treatment, is a highly promising lead to improve the rapidity of scoring. This led to an application to the EPA for continuation of this research, which is currently funded as EPA Grant No. R-808309, and the research continues.
6) The preliminary finding that monoclonal antibodies can be prepared that react with various transformed clones, is extremely promising. The antibodies can be radioiodinated and rapid, objective, and highly sensitive radioimmunoassay can be developed for transformation. Alternatively, the antibody can be labeled with fluorescence and transformed cells can be visualized in a fluorescence microscope and can be separated in a fluorescence activated cell sorter. This is also currently funded on EPA Grant No. R-808309, and the research continues.
7) Although activated mouse peritoneal macrophages selectively kill chemically transformed cells, it is highly unlikely that this test can be made sufficiently sensitive to be used as a primary means of scoring for transformation.

The successes of the approaches described in 1, 5, and 6, together with interesting data collected from the research described in 2, 3, 4, and 7 indicate that the results obtained on this grant, R-805-208, were very successful.

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***Michael D. Waters** is the EPA Project Officer (see below).*
The complete report, entitled "Improved Scoring of Chemical Transformation of C3H/10T1/2 Cells," (Order No. PB 81-209 686; Cost: $6.50, subject to change) will be available only from:
National Technical Information Service
5285 Port Royal Road
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