to those for external ionizing radiation. Thus, the decays of ^3H in DNA do not appear to cause amounts of breakage or irreparable breaks greater than those expected for β particle irradiation.

J. E. CLEAVER
G. H. THOMAS
Laboratory of Radiobiology, University of California, San Francisco 94122
H. J. BURKI
Donner Laboratory, University of California, Berkeley 94720

References and Notes

1. *Biological Effects of Transmutation and Decay of Incorporated Radioisotopes* (International Atomic Energy Agency, Vienna, 1968); E. P. Cronkite *et al.* (Committee 24 of the National Council on Radiation Protection and Measurements), *Radioactive Nucleic Acids and Precursors*, in preparation.
2. L. E. Feinendegen, *Tritium-Labeled Molecules in Biology and Medicine* (Academic Press, New York, 1967); J. E. Cleaver, *Thymidine Metabolism and Cell Kinetics* (North-Holland, Amsterdam, 1967).
3. G. Marin and M. A. Bender, *Int. J. Radiat. Biol.* **7**, 235 (1963); *ibid.*, p. 221; H. J. Burki and S. Okada, *Biophys. J.* **8**, 445 (1968).
4. S. Apelgot and M. Duquesne, *Int. J. Radiat. Biol.* **7**, 65 (1963); S. Apelgot, *ibid.* **10**, 495 (1966); R. Bockrath, S. Person, F. Funk, *Biophys. J.* **8**, 1027 (1968).
5. S. Person and H. L. Lewis, *Biophys. J.* **2**, 451 (1962); S. Person, in *Biological Effects of Transmutation and Decay of Incorporated Radioisotopes* (International Atomic Energy Agency, Vienna, 1968), p. 29.
6. D. E. Wimber, *Proc. Nat. Acad. Sci. U.S.A.* **45**, 839 (1959); J. G. Brewen and G. Olivieri, *Radiat. Res.* **28**, 779 (1966); W. C. Dewey, R. M. Humphrey, B. A. Jones, *ibid.* **24**, 214 (1965).
7. P. M. Rosenthal and M. S. Fox, *J. Mol. Biol.* **54**, 441 (1970).
8. D. R. Hall, J. D. Lewis, C. D. Town, P. J. Fish, P. J. Lindop, *Int. J. Radiat. Biol.* **16**, 43 (1969).
9. The survival curve of V79 cells irradiated by ^3H decays in DNA at −196°C is given by an extrapolation number of 5 and a D_0 dose of 1100 decays per cell (H. J. Burki, R. Roots, L. E. Feinendegen, in preparation).
10. J. E. Cleaver, G. H. Thomas, J. E. Trosko, J. T. Lett, *Exp. Cell. Res.*, in press.
11. J. T. Lett, I. Caldwell, C. J. Dean, P. Alexander, *Nature* **214**, 790 (1967); C. J. Dean, M. G. Ormerod, R. W. Serianni, P. Alexander, *ibid.* **224**, 1042 (1969); A. R. Lehmann and M. G. Ormerod, *Biochim. Biophys. Acta* **204**, 128 (1970); J. T. Lett and C. Sun, *Radiat. Res.* **44**, 771 (1970).
12. For these estimates we assume that M_w is equal to twice the number-average molecular weight for random distributions (*11*), the average DNA content of V79 cells in exponential growth is 8×10^{-12} g, and the nuclear volume is 190 ± 20 μm^3 (D. W. Ross and H. C. Mel, *Biophys. J.*, in press). Errors for breakage efficiency and dose per decay are derived from the slope of regression lines.
13. If the energy from ^3H decays originating in DNA thymine is uniformly distributed in the cell nucleus (*12*), a ^3H decay with average energy of 5.7 kev deposits 0.38 rad in a nucleus of density 1.0 if allowance is made for an edge effect of 20 percent (*2*). The actual volume of frozen cells may be slightly less than 190 μm^3 because of shrinkage during freezing, and the dose of 0.38 rad per decay is thus a minimum estimate. No allowance is made here for the relative biological effectiveness of β particles from ^3H, but current estimates suggest that it is close to 1 in relation to 250 kv-peak x-rays (*2*).
14. B. S. Strauss, *Radiat. Res.* **8**, 234 (1958).
15. M. G. Ormerod and U. Stevens, *Biochim. Biophys. Acta* **232**, 72 (1971).
16. Supported by the Atomic Energy Commission.

24 April 1972

Control of Carcinogenesis: A Possible Role for the Activated Macrophage

Abstract. *Cytotoxic activity of activated mouse macrophages against mouse embryo fibroblasts was tested before and after spontaneous transformation of the fibroblasts in vitro. Activated macrophages caused little or no destruction of untransformed fibroblasts but were markedly cytotoxic to the same fibroblasts after spontaneous transformation. The efferent limb of this cytotoxic reaction appears to be nonimmunologic and to be related to abnormal growth properties rather than to the antigenic composition of target cells.*

When normal mammalian cells are serially cultivated they retain their normal characteristics for a limited period; during this time they are termed a cell strain (*1*). Cell strains are either lost after a variable number of subcultivations (*2*), or they spontaneously develop altered morphology, karyotype, and growth properties (which include unlimited capacity for multiplication) characteristic of cell lines (*3*, *4*). The ease of spontaneous establishment of a cell line appears to be species-dependent; spontaneous establishment of a cell line from a human cell strain rarely if ever occurs (*2*), while in practically all cases murine cell strains develop spontaneously into cell lines within 3 months of culture (*3*, *4*). When cultivated under conditions of extensive cell-to-cell contact, spontaneously established lines of mouse cells usually have, in addition to abnormal in vitro growth properties, in vivo malignant potential (*4–6*). We have reported that activated peritoneal macrophages from mice, unlike normal macrophages, appear to cause in vitro the selective destruction of cells with abnormal growth properties—that is, tumor cells and a cell line—by a nonimmunologic mechanism (*7–9*). We have suggested that the activated macrophage may have a homeostatic role in destroying cells that develop abnormal growth properties in vivo (*9*, *10*).

In the experiments reported here we used mouse fibroblasts as target cells, before and after spontaneous transformation, and showed an altered in vitro reactivity of the activated macrophages to newly established lines of mouse fibroblasts. We report that the cytotoxocity of activated C3H/HeJ macrophages for fibroblast target cells appeared to be related to the acquisition of abnormal growth properties by the fibroblasts, which include loss of contact inhibition of cell division, rather than to antigenic differences between the activated macrophages and target cells. Activated C3H/HeJ macrophages did not destroy allogeneic fibroblast cell strains that have cell surfaces of high immunogenic potential, but were markedly cytotoxic to both syngeneic and allogeneic cell lines. We propose that the cytotoxic effect of activated macrophages for fibroblast cell lines that have developed under subcultivation conditions of extensive cell-to-cell contact may reflect a fundamental host reaction to abnormal cell growth.

Primary cultures of mouse fibroblasts were prepared from 17- to 19-day embryos by the method of Reinhold (*11*). Cells were cultured in Eagle's minimal medium with Earle's salts, streptomycin (100 μg/ml), penicillin (100 unit/ml), and 10 percent fetal calf serum (Gibco, Berkeley, California). All cultures were maintained on a rigid transfer schedule in 950-ml prescription bottles, subcultures at a 1 : 2 ratio being made every 7 days. Medium was changed on day 3. Cells were detached for transfer by adding 0.25 percent trypsin to the monolayers and incubating for 15 minutes at 37°C. Action of trypsin was stopped by adding 4 ml of medium containing serum. When spontaneous transformation to a cell line occurred, changes in morphology and growth characteristics included loss of fusiform shape and parallel orientation of the fibroblasts, cell overgrowth, increased saturation density, and ability to grow at low inoculation density. By these criteria, cell lines were present by subculture 16 in the case of C57BL/6J fibroblasts, by subculture 17 for BALB/c fibroblasts, and by subculture 20 for C3H/HeJ fibroblasts.

To activate macrophages, female C3H/HeJ mice were chronically infected with *Toxoplasma gondii* (*7*) or treated with complete Freund's adjuvant

as described (*8*). Medium 199 (Gibco) with 20 percent fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 unit/ml) was used for incubation of cells in the cytotoxicity test.

Table 1 gives results obtained when target cells were mouse fibroblast cell strains that had been growing for one to eight passages in vitro. In all cases activated macrophages caused little or no cytotoxicity to the fibroblast cell strains, while they were cytotoxic for the L cell line and mouse mammary adenocarcinoma cells (EMT-6 cells). Table 1 also shows results for the same mouse fibroblasts after they had spontaneously transformed to cells with the altered morphology and growth properties of a cell line. Activated macrophages produced a marked cytopathic effect in the newly established fibroblast cell lines. The cytopathic effect was equal to or greater than that seen when L cells or EMT-6 cells were target cells. However, activated macrophages caused little or no damage to freshly prepared strains of fibroblasts which had been growing in vitro for eight passages or less. Figure 1 shows the effect of normal and activated C3H/HeJ ($H\text{-}2^k$) macrophages on a C57BL/6J ($H\text{-}2^b$) cell strain and a C57BL/6J cell line.

It is not likely that the destruction of tumor cells and cell lines mediated by activated macrophages is merely a function of prolonged growth of the target cells in tissue culture; activated macrophages had a marked cytopathic effect on primary cultures of mouse sarcoma cells and EMT-6 cells (*8*). (The mouse L cell line used in our in vitro cytotoxicity studies was found to be free of mycoplasma contamination in the laboratory of L. Hayflick, Stanford University.)

In order to document differences in the in vitro growth properties of the cell strains and cell lines studied (Table 1), growth at low inoculation densities and the cumulative increase in number of cells per plate were measured at the same point in time that the cytotoxicity test was performed on other cells from the same lines and strains. Cells from healthy confluent monolayers were subcultivated in 60-ml plastic petri dishes (Falcon Plastics, Los Angeles, California) at varying dilutions and in duplicate. The medium was changed on day 3, and the cells were grown for 3 more days and then detached with trypsin and counted. At an inoculum density of 1×10^4 cells per plate, there was no net growth of any of the cell strains but all the cell lines were capable of growing maximally (Table 2). There was a significant difference between the growth potential of cell strains and cell lines at all inoculum densities tested (up to 1×10^6 cells per plate). Increased in vitro growth potential correlated well with target cell destruction by activated macrophages.

The spontaneous conversion of fibroblasts to cells with abnormal growth properties eliminates the added variable of an exogenous carcinogen and may be more representative of spontaneous development of neoplasia in situ. Aaronson and Todaro (*6*) demonstrated that spontaneous loss of contact inhibition of cell division by mouse fibroblasts in vitro correlates well with tumorigenicity.

We found that activated C3H/HeJ macrophages did not destroy two allogeneic fibroblast cell strains that differed from them at the strongly antigenic H-2 locus. In contrast, these macrophages were markedly cytotoxic for the same allogeneic fibroblasts (and also for syngeneic fibroblasts) after the fibroblasts had spontaneously developed abnormal growth properties that included loss of contact inhibition of cell division. Because H-2 antigens are more immunogenic than tumor-specific transplantation antigens (*12*), this study strongly suggests that a change associated with the acquisition of abnormal growth properties, rather than a change in antigens, is probably responsible for the altered reactivity of the activated macrophage (*8, 9*). There is evidence that the genetic information for C-type virus production is present in every mouse cell (*13*), and that some of the mouse fibroblast cell lines that develop spontaneously in culture begin to release C-type virus. Virus production de-

Table 1. Action of activated macrophages on mouse fibroblasts before and after spontaneous transformation of fibroblasts in vitro; 0, little or no target cell destruction; +, marked target cell destruction. Peritoneal cells (2×10^6), obtained as described (*16*), were pipetted onto sterile cover slips that had been placed in 35 by 10 ml tissue culture dishes (Falcon Plastics). The dishes were incubated for 2 hours at 37°C in air containing 5 percent CO_2 to allow for adherence of cells. The cover slips were washed five times with a total of 20 ml of normal sterile 0.15*M* NaCl to remove nonadherent cells. Target cells and their preparation have been described (*8*). A suspension of target cells, 1×10^5 cells in 0.1 ml of medium, was added to cover slips containing the washed monolayers of adherent peritoneal cells (macrophages) from which medium had been removed. Additional medium was added to dishes after target cells were allowed to settle onto the adherent peritoneal cells for 20 minutes, and the tissue culture dishes containing mixed macrophages and target cells were incubated for 60 hours at 37°C in atmosphere containing 5 percent CO_2. The cover slips were then fixed in methanol and stained with Giemsa stain. Purity of macrophages in control monolayers was determined to be greater than 99 percent by the criterion of phagocytosis of heat-killed *Candida albicans*.

Target cells	Source of effector cells [C3H/HeJ ($H\text{-}2^k$) peritoneal macrophages]		
	Chronic *Toxoplasma gondii* infection	Treated with complete Freund's adjuvant	Control
Before transformation			
C3H/HeJ fibroblast strain ($H\text{-}2^k$)	0	0	0
BALB/c fibroblast strain ($H\text{-}2^d$)	0	0	0
C57BL/6J fibroblast strain ($H\text{-}2^b$)	0	0	0
L cell ($H\text{-}2^k$)	+	+	0
EMT-6 mammary adenocarcinoma ($H\text{-}2^d$)	+	+	0
*After transformation**			
C3H/HeJ fibroblast cell line	+	+	0
BALB/c fibroblast cell line	+	+	0
C57BL/6J fibroblast cell line	+	+	0

* No destruction was noted of freshly prepared C3H/HeJ, BALB/c, and C57BL/6J cell strains used as controls for experiments with spontaneously formed cell lines.

Table 2. Cell strain and cell line growth kinetics. Results are given as the ratio of the number of cells in culture after 6 days to the number of cells inoculated (S, cell strain; L, cell line).

Cells	Number of cells inoculated							
	5×10^4		1×10^5		5×10^5		1×10^6	
	S	L	S	L	S	L	S	L
L cell		20		24		7.4		5.0
EMT-6		48		29		6.2		5.1
C3H/HeJ	0	29	1.5	23	3.6	9.0	3.6	5.9
BALB/c	5	32	8.5	27	3.6	10.4	3.6	8.5
C57BL/6J	7	37	6.5	22	3.7	11.5	3.2	7.5

veloped only in cells that had lost normal control of growth (*14*). It is possible that in our experiments the activated macrophage is reacting to C-type virus. This explanation for the reactivity of activated macrophages against mouse cell lines is probably insufficient, because the spontaneous development by cells of abnormal growth properties in vitro is not always associated with the release of C-type virus (*14*).

We showed earlier that mice having a population of activated macrophages with increased in vitro cytotoxic capability (*8*, *9*) also have increased resistance to autochthonous and transplanted tumors (*7*, *10*, *12*, *15*). These results, coupled to those reported here for the in vitro system, provide further evidence that the activated macrophage may have a role in the control of newly emergent cells with abnormal growth properties in vivo (*8*, *9*).

JOHN B. HIBBS, JR.*
LEWIS H. LAMBERT, JR.
JACK S. REMINGTON
Division of Allergy, Immunology, and Infectious Diseases, Palo Alto Medical Research Foundation, Palo Alto, California 94301, and *Division of Infectious Diseases, Department of Medicine, Stanford University School of Medicine, Palo Alto 94305*

References and Notes

1. We define a cell strain and a cell line according to the criteria of Hayflick and Moorhead (*2*), with the added qualification that we are referring in this report to a specific type of cell line, one that has lost contact inhibition of cell division. By these criteria, all cell strains have limited capacity for multiplication and normal growth properties, while cell lines have an unlimited capacity for multiplication and in addition may exhibit one or more abnormalities of in vitro cell growth, which include density inhibition of growth (contact inhibition of cell division) [M. G. P. Stoker and H. Rubin, *Nature* **215**, 171 (1967)].
2. L. Hayflick and P. S. Moorhead, *Exp. Cell Res.* **25**, 585 (1961).
3. G. J. Todaro and H. Green, *J. Cell Biol.* **17**, 299 (1963).
4. K. H. Rothfels, E. B. Kupeliwieser, R. C. Parker, *Proc. Can. Cancer Res. Conf.* **5**, 191 (1963).
5. W. R. Earle and A. Nettleship, *J. Nat. Cancer Inst.* **4**, 213 (1943).
6. S. A. Aaronson and G. J. Todaro, *Science* **162**, 1024 (1968).
7. J. B. Hibbs, Jr., L. H. Lambert, Jr., J. S. Remington, *J. Clin. Invest.* **50**, 45a (1971).
8. ———, *Nature New Biol.* **235**, 48 (1972).
9. ———, *Proc. Soc. Exp. Biol. Med.* **139**, 1049 (1972).
10. ———, *ibid.*, p. 1053.
11. H. S. Reinhold, *Eur. J. Cancer* **1**, 67 (1965).
12. R. T. Prehn and J. M. Main, *J. Nat. Cancer Inst.* **18**, 769 (1957).
13. S. A. Aaronson, G. J. Todaro, E. M. Scolnick, *Science* **174**, 157 (1971).
14. S. A. Aaronson, J. W. Hartley, G. J. Todaro, *Proc. Nat. Acad. Sci. U.S.A.* **64**, 76 (1969).
15. J. B. Hibbs, Jr., L. H. Lambert, Jr., J. S. Remington, *J. Infect. Dis.* **124**, 587 (1971).
16. J. Ruskin and J. S. Remington, *Science* **160**, 72 (1968).
17. Supported by NIH special postdoctoral research fellowship 43421 to J.B.H. and by a grant from the John A. Hartford Foundation.
* Present address: Division of Infectious Diseases, Department of Internal Medicine, University of Utah College of Medicine, Salt Lake City 84112.

27 April 1972

Genetic Meaning of Zooecial Chamber Shapes in Fossil Bryozoans: Fourier Analysis

Abstract. *Fourier harmonic amplitudes quantitatively characterize chamber shapes of fossil tubular bryozoans. The odd-numbered harmonics, particularly the seventh, carry evolutionary information. The phenotypically plastic second and sixth harmonics measure zooecial orientation and packing, respectively. As a measure of crowding, the sixth harmonic reflects mechanistic growth response to paleoenvironmental conditions.*

Fourier analysis quantifies shape differences among zooecial chambers of fossil bryozoans. Genetics, growth, orientation, and packing control shape components. The sixth harmonic reflects packing and gross colony form; the second reflects orientation and, in ramose forms, ontogeny. Distance from a monticular center controls the fourth, fifth, and sixth harmonics. The other harmonics vary little within a colony, but widely among evolving taxa.

Ehrlich and Weinberg (*1*) have shown that two-dimensional closed shapes can be characterized to any desired degree of precision by using the harmonic coefficients of the Fourier series as shape descriptors (*2*). Younker (*3*) and Kaesler and Waters (*4*) have demonstrated the utility of Fourier harmonics in the study of ostracode carapaces. Each harmonic makes an independent contribution to the total shape: The first describes the contribution of an offset circle, the second of a figure eight, the third of a trefoil, and the fourth of a quatrefoil. The *n*th harmonic describes the contribution made to a given shape by a "clover" with *n* leaves. All equilateral polygons with *n* sides yield a high amplitude in the *n*th harmonic and in higher harmonics that are multiples of *n*.

Harmonic spectra from tangential sections of a large hemispherical colony of *Amplexopora filiasa* (Fig. 1) show considerable shape variation among genetically identical zooids (*5*). The chambers fall into three shape families: monticular zooecia (megazooecia), zooecia in the aureole surrounding each monticule (*6*), and zooecia of the intermonticular areas. Megazooecia are generally quadrate, and so have a high fourth harmonic. Zooecia in the monticular aureole are generally pentagonal, displaying a high fifth harmonic. Intermonticular zooecia, however, most commonly hexagonal, have a high sixth harmonic (Fig. 2A). The second harmonic also varies within a colony. Megazooecia and aureole zooecia are both more elongate than intermonticular zooecia, and so have higher second harmonics. Their elongation results from the elevation of the monticule above the colony surface, forming a wartlike protuberance in which the tubes are inclined to the colony surface and therefore appear elongate in section. The second harmonic measures the deviation from the perpendicular. In variously oriented cross sections of the same zooecial prism, only the second harmonic changes; the others remain constant.

When most densely packed, circular cylinders pressed together will become hexagonal prisms (*7*), and their amplitude spectra will display prominent sixth harmonics. Intracolonial spectra (Fig. 2A) suggest that packing is lowest in the monticules and increases toward the intermonticular regions; the spectra show a radial packing gradient corresponding to a decrease in zooecial diameter. This gradient, and the space-filling structures (denser wall material and "mesopores") in the monticular centers (Fig. 1), suggest that monticules represent the budding centers of the colony, and that the packing gradient indicates successive stages of zooecial ontogeny. As the cylinders extend themselves distally, new zooids are budded interstitially in the monticules. As more zooids are budded, they are pushed away from the monticule into the monticular aureole, and finally into the densely packed intermonticular area. In ontogeny, the zooids decrease in size, change orientation, and develop additional sides until their cross sections ultimately become equilateral hexagons.

The mean harmonics of different colonies illustrate the variation among different taxa and growth forms (Fig. 2B). The amplitude spectra of three