was aerosolized in the pneumatic cleaning process. In this outbreak, however, the relative importance of direct contact or aerosolization in spread cannot be determined.

The earliest outbreak of legionellosis documented so far appeared to result from airborne spread from sites of excavation of soil (*13*), and sporadic cases of legionellosis have been associated with excavation and construction (*14*). It may be that *L. pneumophila* is basically a soil organism, the ecologic niche and epidemic potential of which are expanded by the increasing use of water in various heat-rejection systems.

What determines whether *L. pneumophila* will cause Legionnaires' disease or Pontiac fever is obscure. It is unlikely that dose of organism alone suffices to explain the difference, since one would expect the disease with the larger dose to have both the shorter incubation period and the more severe course. Perhaps Pontiac fever results from a large dose of nontoxigenic organisms, but no differences of obvious importance—including presence of toxins—have been found in laboratory testing of strains of *L. pneumophila* that caused Legionnaires' disease or Pontiac fever. Eickhoff has suggested that Pontiac fever results from exposure to a large number of dead *L. pneumophila* organisms (*6*). Although an attractive hypothesis, it does not explain the success in recovering live *L. pneumophila* from the lungs of guinea pigs that developed pneumonia following exposure in the building where the Pontiac fever epidemic occurred (*3*).

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Cycloheximide-Dependent Reversion of Human Cells Transformed by MSV and Chemical Carcinogen

Abstract. *The protein synthesis inhibitor cycloheximide, at a concentration of 0.08 microgram per milliliter, induced flat morphology within 24 to 48 hours and low saturation density in human osteosarcoma cells transformed by Kirsten murine sarcoma virus (Ki-MSV) or* N-*methyl*-N′-*nitro*-N-*nitrosoguanidine. Removal of the protein synthesis inhibitor caused both transformed cells to revert to the transformed phenotype. The demonstration of cell-surface antigens, cross-reacted with antiserums induced by extracts of both types of transformed human cells, was dependent on the presence or absence of cycloheximide in the culture medium. The results show that protein synthesis is required to maintain the transformed state in virally or chemically transformed human cells.*

Flat morphology and low saturation density of cells studied in vitro are often regarded as markers of normal behavior. Cells transformed by oncogenic DNA and RNA viruses or by chemical carcinogens can revert to variant forms in which their morphology and function resemble those of normal cells (*1*-*5*). In attempts to understand the basis for contact inhibition of mitosis, a number of agents, including inhibitors of protease and hyaluronidase enzymes, have been used to induce flat morphology in cultured transformed cells (*6*, *7*). However the mechanism of reversion is not known. Recently, Krzyzek *et al.* (*8*) observed that revertant subclones of cells infected with Rous sarcoma virus (RSV) contain as much sarcoma-specific RNA as the transformed cells from which they were derived. This suggests that reversion might be caused by a posttranscriptional restriction of the expression of the viral transforming gene or genes. Addition of protein synthesis inhibitors to rat kidney cells transformed by a temperature-sensitive mutant of RSV at a permissive temperature caused the cells to revert temporarily to normal phenotype (*9*). This finding implies that protein synthesis, presumably of an unstable product of the transforming gene of the temperature-sensitive virus, is required to maintain the transformed state in these infected cells at the permissive temperature. Recently a transformation-specific antigen from cells transformed by avian sarcoma virus has been identified (*10*).

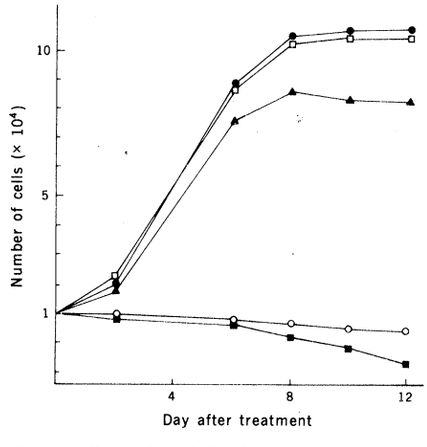

Fig. 1. Effect of cycloheximide on the growth of human osteosarcoma TE-85, clone F-5, cells (●) grown in EMEM plus 10 percent FBS medium without cycloheximide or in the medium containing cycloheximide at (□) 0.08 μg/ml, (▲) 0.1 μg/ml, (○) 1.0 μg/ml, or (■) 10 μg/ml.

In view of these results, we reasoned that inhibition of protein synthesis might affect the rate of growth and morphology of human cells transformed by oncogenic viruses. Further, chemically transformed human cells could also revert to an untransformed state by restricting the expression of the transforming gene or genes. In this report we present evidence that morphologic reversion and selective inhibition of growth can be observed in

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Table 1. Effect of cycloheximide on growth and phenotype of human osteosarcoma (HOS) cells and Ki-MSV- or MNNG-transformed HOS cells.

Cell line	Cycloheximide (0.08 μg/ml) in medium	Morphology	Saturation density* ($\times 10^5$ cm^{-2})	Cell aggregates†		Plating efficiency in soft agar‡ (%)
				Size	Viability of cells ($\times 10^5$)	
Ki-MSV-transformed	No	Transformed	2.5	Large	6.7	8.6
Ki-MSV-transformed	Yes	Flat	0.22	Small	1.4	2.1
MNNG-transformed	No	Transformed	2.90	Large	4.2	7.2
MNNG-transformed	Yes	Flat	0.26	Small	1.3	1.9
HOS control	No	Flat	0.28	Small	1.7	1.2
HOS control	Yes	Flat	0.15	Small	1.5	1.0

*Maximum number of cells obtained after initially plating with 5×10^3 cells per square centimeter and incubating at 36°C under conditions where growth medium (EMEM plus 10 percent FBS) was changed every 3 days. †Cell aggregates formed after 4 days in an agar static system; viability of cell aggregates was determined 4 days after plating 2×10^6 cells per plate initially. ‡Colony-forming efficiency (percent) = (number of colonies formed divided by number of cells plated) × 100.

virally or chemically transformed human cells after treatment with cycloheximide, a chemical that inhibits protein synthesis.

The human osteosarcoma (HOS) cell line TE-85, clone F-5, originally established by McAllister *et al.* (*11*), and the HOS cell lines transformed by Kirsten murine sarcoma virus (Ki-MSV) (*12*) or by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (*13*) were used in this study. These cell lines have been described in detail. Cells were grown and maintained in Eagle's minimum essential medium (EMEM) with 10 percent fetal bovine serum (FBS), 2 m*M* glutamine, 100 units of penicillin, and 100 μg of streptomycin per milliliter (EMEM plus 10 percent FBS). Cycloheximide (Sigma Chemical Co.) was dissolved directly in the medium and diluted before use.

As one parameter of cytotoxicity, the effect of cycloheximide on cell growth was investigated. Untransformed HOS cells were plated in 60-mm Falcon plastic plates with medium containing various concentrations of cycloheximide and incubated at 36°C; the growth medium plus cycloheximide was changed every 3 days. As shown in Fig. 1, at a cycloheximide concentration of 0.08 μg/ml no substantial inhibition in growth was observed, as indicated by the fact that the doubling time was identical to that measured for cells grown in medium without cycloheximide. Some toxicity, determined as inhibition of cell growth, was found at a cycloheximide concentration of 1 μg/ml, and at 10 μg/ml all growth was completely inhibited (Fig. 1). Protein synthesis was also measured by incorporation of L-[^{3}H]leucine (New England Nuclear) into trichloroacetic acid-insoluble material. Cultures were first treated with cycloheximide for 1 hour and then incubated with the labeled amino acid in medium plus cycloheximide for an additional 4 hours. At a cycloheximide concentration of 10 μg/ml protein synthesis was inhibited by 95 percent; however, at 0.08 μg/ml protein synthesis was reduced by only 50 percent. Therefore, a cycloheximide concentration of 0.08 μg/ml was used for further experiments with transformed HOS cells.

Addition of 0.08 μg of cycloheximide per milliliter to the culture medium caused Ki-MSV- or MNNG-transformed HOS cells to profoundly alter their morphology (Fig. 2). The cells became flat within 24 to 48 hours and there was no overlapping. This resulted in a monolayer of low cell density and a cobblestone appearance, as illustrated in Fig. 2. Removal of the protein inhibitor caused both transformed cell lines to revert to the transformed phenotype. Transformed cells in medium containing cycloheximide at 0.08 μg/ml could be maintained in the flat morphology without cytotoxic effect for six subcultures. However, those treated cells could be reverted to the transformed state when the cycloheximide was removed from the medium. The flattening effect was also reflected in the growth curves of treated cells. As shown in Table 1, the saturation density of both transformed cells was strikingly reduced, and the cells aggregated poorly above an agar layer (*14*). Counts of viable cells in trypsinized aggregates on four consecutive days showed a significant decline in transformed cells treated by cycloheximide, whereas transformed cells in the absence of cycloheximide exhibited growth in the aggregate form. The plating efficiency of cycloheximide-treated transformed cells in soft agar was less than that of transformed cells without cycloheximide (Table 1).

To determine whether cell surface antigens on both transformed cells could be demonstrated, the treated cells were examined by indirect immunofluorescence. Antiserums, twice absorbed by the HOS cells, were obtained from Fischer rats by immunization with clarified extracts of

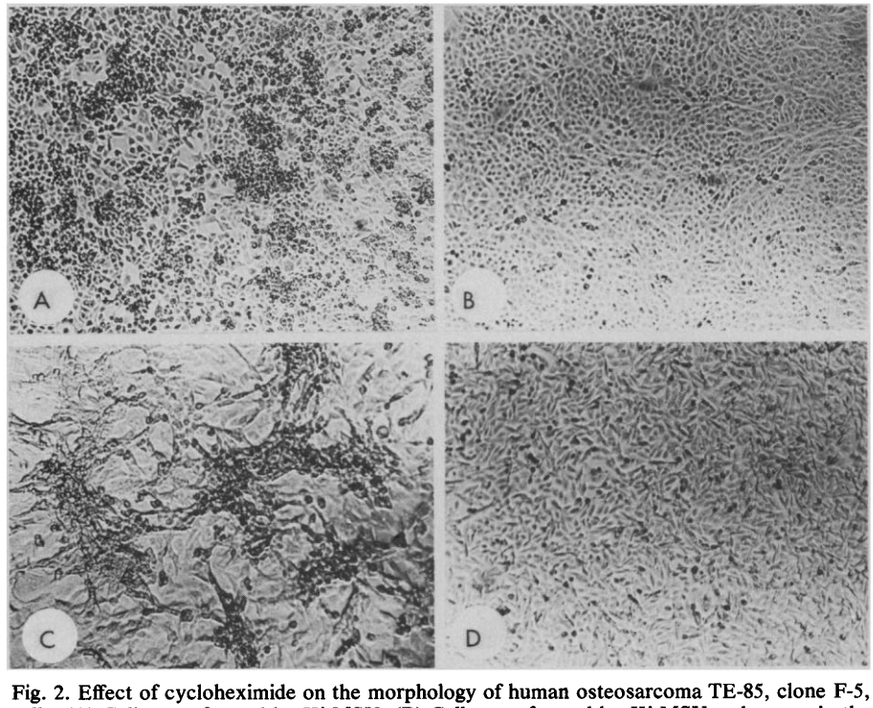

Fig. 2. Effect of cycloheximide on the morphology of human osteosarcoma TE-85, clone F-5, cells. (A) Cells transformed by Ki-MSV. (B) Cells transformed by Ki-MSV and grown in the presence of cycloheximide (0.08 μg/ml) for 8 days. (C) Cells transformed by MNNG. (D) Cells transformed by MNNG and grown in the presence of cycloheximide (0.08 μg/ml) for 8 days.

virally and chemically transformed HOS cells. Fluorescein-conjugated rabbit antiserum to rat immunoglobulin G (Microbiological Associates) was used at a dilution of 1:15. Indirect immunofluorescence in cultures of both types of transformed cells, treated with cycloheximide or not, was scarcely visible with their antiserums. Immunofluorescent staining of proteins on the surfaces of both types of transformed cells could be observed. However, the cell surface antigens could not be detected in virally or chemically transformed HOS cells treated with cycloheximide. The immunofluorescence index (*15*) was less than 30 percent (considered negative) in both types of treated cells.

Our results demonstrate that the addition of cycloheximide (0.08 μg/ml) in culture medium caused both virally and chemically transformed human osteosarcoma cells (TE-85, clone F-5) to revert temporarily to normal phenotype, but the same amount of cycloheximide did not affect the rate of growth of untransformed cells. Increasing the amount of inhibitor from 0.08 to 1 μg/ml strongly inhibited normal cell growth, suggesting that the primary effect of this protein inhibitor on both types of transformed cells may be one of nonspecific toxicity. The mechanism by which cycloheximide maintains the flat morphology in the transformed cells is not understood. Regulation of the expression of either oncogenic RNA virus or chemical carcinogen transforming gene sequences might be under the influence of a translational control mechanism because of a translational inhibitor-dependent reversion in both types of transformed cells. Demonstration of surface antigens, cross-reacted with antiserums induced by extracts of both types of transformed cells, also depended on the presence or absence of cycloheximide in the culture medium. These cell surface antigens (*16, 17*) are clearly associated with the process of transformation, and whatever mechanism was involved in the transformation, the malignant state could be reversed by an inhibitor of protein synthesis. These results show that protein synthesis is required to maintain the transformed state in chemically and virally transformed human cells. Synthetic inhibitors of proteases have been used successfully to suppress 7,12-dimethylbenz[*a*]anthracene-induced and phorbol ester-promoted tumorigenesis in mouse skin (*18*). The possibility of accomplishing the same thing in vivo would have exciting implications for the management of human cancers.

Cycloheximide has also been shown to induce type C virus at high frequency from virus-negative mouse cells by an increase in the cellular concentration of type C viral RNA in cycloheximide-treated cells (*19*). However, no type C virus induction was observed in the cycloheximide-treated chemically altered human cells.

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Cerebral Cortical Microfluorometry at Isosbestic Wavelengths for Correction of Vascular Artifact

Abstract. *Microfluorometric measurements of cerebral cortical mitochondrial respiration in vivo are obscured by hemodynamic and oximetric artifacts. Isosbestic fluorometry provides appropriate correction for these vascular phenomena and permits simultaneous evaluation of mitochondrial nicotinamide adenine dinucleotide redox state, microcirculatory volume, and hemoglobin oxygenation.*

Tissue microfluorometry was originally introduced as an optical method whereby mitochondrial respiration could be examined continuously and nondestructively in vivo (*1*). The transducible property underlying the technique is the intense blue fluorescence (440 to 470 nm) produced when reduced mitochondrial nicotinamide adenine dinucleotide (NADH) is excited by light in the near-ultraviolet portion of the spectrum (340 to 370 nm); the oxidized cofactor (NAD), as well as unbound or cytoplasmic NADH, exhibits negligible fluorescence (*1, 2*). This property was initially exploited in studies of the NADH : NAD redox state in isolated mitochondria by Chance *et al.* in the early 1950's, and soon thereafter microfluorometry was applied to metabolic studies of intact tissue in situ (*1*). Subsequently, numerous investigators have variously modified the method to examine mitochondrial behavior in heart, liver, kidney, thyroid, and brain (*3*).

Cerebral microfluorometry, developed most fully by Jobsis and co-workers (*4*), has been used to provide a qualitative indication of the kinetics and efficiency of cortical mitochondrial respiration during a variety of experimental interventions in both laboratory animals and in humans (*5*). On review, it is apparent that tissue microfluorometry is no longer a single instrumentation system as initially described by Chance *et al.* but a family of distinct systems, each based on the same transducible property (NADH fluorescence), yet each reflecting a distinct opinion concerning significant technical problems that compromise fluorometric analysis in situ. These problems appear to be inherent in tissue microfluorometry and have produced increasing uncertainty over both the proper application of the method and its utility in general (*6*).

Persuaded that the potential of the fluorometric method is not yet fully realized, we have studied in considerable detail the optical properties of cerebral cortical tissue in order to identify and eliminate the artifacts that most seriously compromise the use of fluorometry in

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