transducin by rhodopsin leads to an extremely rapid decrease in cyclic GMP concentrations in the cell which may affect sodium ion permeability in the rod outer segment plasma membrane. The *ras* gene products may be part of a comparable information processing system albeit controlling a different regulatory pathway. There is a growing body of data to suggest that Ras protein mediates signals that regulate cell growth and cell division (*16*). Recent studies have shown that Ras protein is associated with cell surface receptors involved in growth control—for example, the insulin, epidermal growth factor (EGF) (*11*), and possibly transferrin receptors (*17*). The Ras protein may behave like the α subunit of G proteins in that it may cycle through alternative configurations as a result of its association with receptor and guanine nucleotides. Further understanding of the G proteins and the Ras proteins and analysis of the genes that encode them will define in detail the relation between these two gene families.

JAMES B. HURLEY
MELVIN I. SIMON
DAVID B. TEPLOW
Division of Biology,
California Institute of Technology,
Pasadena 91125
JANET D. ROBISHAW
ALFRED G. GILMAN
Department of Pharmacology,
University of Texas Health Science
Center at Dallas, Dallas 75235

References and Notes

1. A. G. Gilman, *Annu. Rev. Biochem.* **49**, 533 (1980); M. Rodbell, *Nature (London)* **284**, 17 (1980).
2. A. G. Gilman, *Cell* **36**, 577 (1984).
3. L. Stryer, J. B. Hurley, B. K.-K. Fung, *Curr. Top. Membr. Transp.* **15**, 93 (1981).
4. P. C. Sternweiss and J. Robishaw, *J. Biol. Chem.*, in press.
5. M. E. Abood, J. B. Hurley, M. C. Pappone, H. R. Bourne, L. Stryer, *ibid.* **257**, 10540 (1982); D. R. Manning, B. A. Fraser, R. A. Kahn, A. G. Gilman, *ibid.* **259**, 749 (1984); C. VanDop *et al.*, *ibid.*, p. 23.
6. D. R. Manning and A. G. Gilman, *ibid.* **258**, 7059 (1983).
7. J. B. Hurley, H. K. W. Fong, D. B. Teplow, W. J. Dreyer, M. I. Simon, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
8. B. K.-K. Fung and C. Nash, *J. Biol. Chem.* **258**, 10503 (1983); K. R. Halliday, P. Stein, N. Chernoff, G. L. Wheeler, M. W. Bitensky, *ibid.* **259**, 516 (1984).
9. M. C. Willingham, I. Pasten, T. Y. Shih, E. M. Scolnick, *Cell* **19**, 1005 (1980).
10. T. Katada and A. G. Gilman, in preparation; T. Y. Shih *et al.*, *Nature (London)* **259**, 516 (1984).
11. T. Kamata and J. R. Feramisco, *Nature (London)* **310**, 147 (1984).
12. E. H. Chang, M. H. Gonda, R. W. Ellis, E. M. Scolnick, D. R. Lowy, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4848 (1982); D. R. Manning and A. G. Gilman, *J. Biol. Chem.* **258**, 7059 (1983); M. W. Bitensky, G. L. Wheeler, A. Yamazaki, M. Rasenick, P. Stein, *Curr. Top. Membr. Transp.* **15**, 237 (1981).
13. C. J. Tabin *et al.*, *Nature (London)* **300**, 143 (1982); K. Shimizu *et al.*, *ibid.* **304**, 497 (1983); D. J. Capon *et al.*, *ibid.*, p. 507.
14. J. P. McGrath, D. J. Capon, D. V. Goeddel, A. D. Levinson, *ibid.* **310**, 644 (1984).
15. N. Gay and J. Walker, *ibid.* **301**, 262 (1983).
16. J. Campisi, H. E. Gray, A. B. Pardee, M. Dean, G. E. Sonenshein, *Cell* **36**, 241 (1984).
17. T. Finkel, C. J. Der, G. Cooper, *ibid.* **37**, 151 (1984).
18. B. K.-K. Fung, J. B. Hurley, L. Stryer, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 152 (1981).
19. M. W. Hunkapiller, E. Lujon, F. Ostrander, L. E. Hood, *Methods Enzymol.* **91**, 227 (1983).
20. M. W. Hunkapiller and L. E. Hood, *ibid.*, p. 486.
21. M. Lochrie, personal communication.
22. E. Taparowsky, K. Shimizu, M. Goldfarb, M. Wigler, *Cell* **34**, 581 (1984); J. P. McGrath *et al.*, *Nature (London)* **304**, 501 (1983); D. Defeo-Jones, E. M. Scolnick, R. Koller, R. Dhar, *ibid.* **306**, 707 (1983); D. Gallwitz, C. Donath, C. Sander, *ibid.*, p. 704.
23. We thank Michael Lochrie for helpful discussions and Rosemary Hopkins for technical assistance. Supported by NIH grant NS 18153 (A.G.G.), American Cancer Society grant BC 240 (A.G.G.), NIH fellowship GM 09731-02 (J.D.R.), and NSF grant PCM 8411039 (M.I.S.).

20 September 1984; Accepted 16 October 1984

Effect of Cell-Cell Interactions on Drug Sensitivity and Growth of Drug-Sensitive and -Resistant Tumor Cells in Spheroids

Abstract. *Multicellular spheroids were grown from mixtures of rat brain tumor cells sensitive (9L) and resistant (R_3) to 1,3-bis(2-chloroethyl)-1-nitrosourea. Percentages of each cell subpopulation in these spheroids were estimated with the sister chromatid exchange assay and were found to be approximately the same as those used to initiate spheroids. Spheroids grown from 9L cells alone had a higher growth rate than spheroids grown from R_3 cells alone. However, the growth rate of mixed-cell spheroids was essentially the same as that of pure 9L spheroids and was independent of the percentages of R_3 cells in mixed-cell spheroids. The sensitivity of 9L cells in mixed-cell spheroids treated with 1,3-bis(2-chloroethyl)-1-nitrosourea, estimated by changes in the number of sister chromatid exchanges per metaphase induced by treatment, decreased as the percentage of R_3 cells increased. These effects are probably the result of an interaction between the two cell subpopulations held in three-dimensional contact, a situation similar to that in tumors in situ. The results suggest why one cell subpopulation of tumors does not become dominant during growth and indicate that interactions between cell subpopulations can influence the sensitivity of one subpopulation to 1,3-bis(2-chloroethyl)-1-nitrosourea.*

Characterization of isolated tumor cell subpopulations has provided important information on the clonal diversity in a tumor and on the basic biology of individual cell subpopulations. However, recent studies (*1*) in which interactions between tumor cell subpopulations were detected suggest that this information may be misleading if attempts are made to discern the behavior of cells in tumors

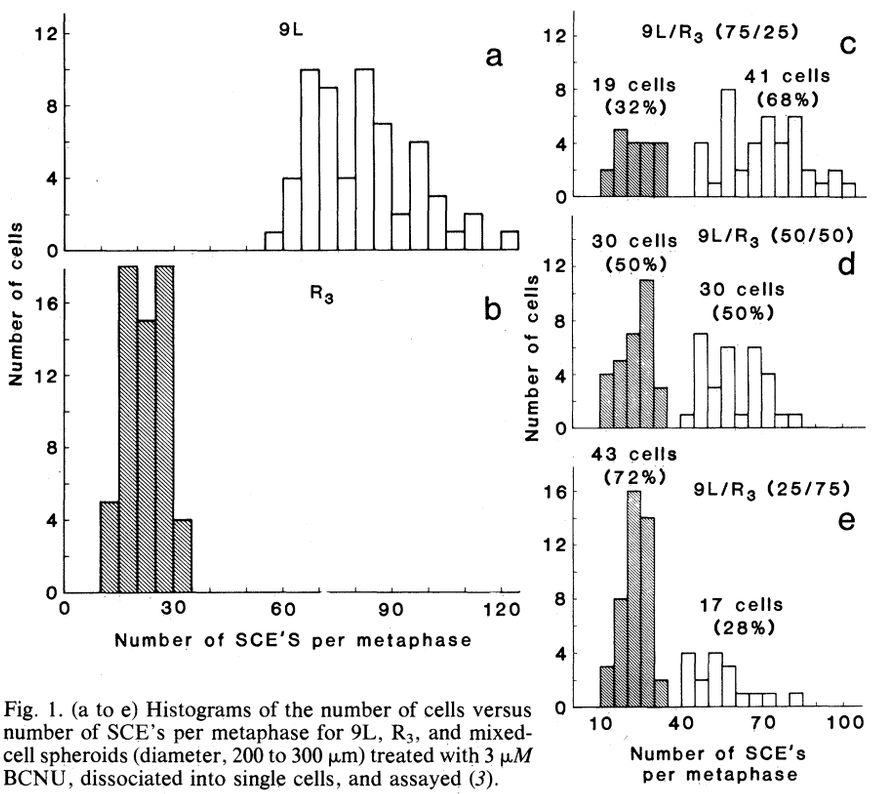

Fig. 1. (a to e) Histograms of the number of cells versus number of SCE's per metaphase for 9L, R_3, and mixed-cell spheroids (diameter, 200 to 300 μm) treated with 3 μ*M* BCNU, dissociated into single cells, and assayed (*3*).

in situ. Interactions between tumor cell subpopulations that affect drug sensitivity and growth rate have been found in vivo and in vitro for cells grown under specific experimental conditions (*1*). However, these interactions have not been reported when different cell subpopulations are grown in three-dimensional contact for extended periods of time, a situation similar to that in tumors in situ. The major obstacle to the study of cell-cell interactions has been the lack of a quantitative method that could distinguish between cell types and could determine the drug sensitivities of each subpopulation.

Multicellular spheroids approximate many characteristics of in vivo tumors that usually are not present in single-cell systems in vitro (*2*), including three-dimensional intercellular contact, ranges in *p*H, oxygen tension, nutrient levels, and the ability to be grown in culture for several weeks without trypsinization. Yet spheroids can be grown under the rigidly controlled environmental conditions common to culture systems in vitro. Spheroids used in this study were initiated and grown from mixtures of rat brain tumor cells sensitive (9L) and resistant (R_3) to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and simulate the heterogeneity, with respect to the drug sensitivity of cells, of solid tumors in situ.

The sister chromatid exchange (SCE) assay is a sensitive, simple method for measuring damage to DNA. Induction of SCE's in 9L cells treated in monolayer culture with BCNU has been directly correlated with cell kill (*3*), and agents such as α-difluoromethylornithine (*4*) and x-rays (*5*) that modify the cell kill caused by BCNU also modify the induction of SCE's by BCNU. Thus in this system the SCE assay provides the same relative indication of cytotoxicity as the cell survival assay. However, because the SCE assay is based on analysis of individual cells, it can be used to distinguish quantitatively between BCNU-sensitive and -resistant cells grown in the same culture. We have reported the results of experiments in which various proportions of BCNU-sensitive and -resistant 9L cells were mixed in monolayer culture and treated with BCNU (*6*). When data obtained with the SCE assay were plotted as histograms representing the number of cells versus the number of SCE's per metaphase, two regions corresponding to the sensitive and resistant populations were obtained, and the approximate percentages of each cell type could be predicted for each mixture. We have now extended these studies to spheroids grown from mixtures of BCNU-sensitive and -resistant 9L cells.

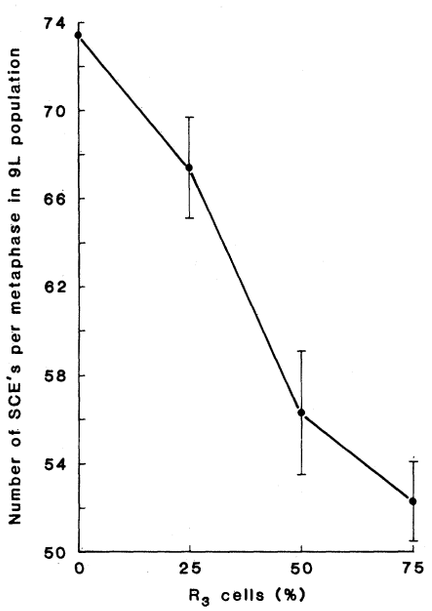

Fig. 2. Plot of the number of SCE's per metaphase in the individual 9L cell populations versus the percentage of R_3 cells. Spheroids were treated for 1 hour with 3 μ*M* BCNU. Values are means ± standard errors for three experiments for spheroids with diameters of 100 to 500 μm.

Table 1. Initial and experimentally determined percentages of 9L and R_3 cells in spheroids. Results are shown for a representative experiment in which spheroids were treated for 1 hour with 3 μ*M* BCNU and 60 SCE's per metaphase were scored for each group of spheroids.

Spheroid diameter (μm)	Initial mixture (9L/R_3)	SCE assay (9L/R_3)
100 to 200	75/25	73/27
	50/50	58/42
	25/75	35/65
400 to 500	75/25	68/32
	50/50	48/52
	25/75	37/63

The BCNU-resistant cell line used in these studies, R_3, was cloned from the resistant line $9L_2$ (*7*). The R_3 cell line has the same response to BCNU as reported for $9L_2$ cells (*6*). Doubling times for R_3 and 9L cells in monolayer culture are each approximately 19 hours; modal chromosome numbers are 57 and 55, and the background levels of SCE's per metaphase are 12.6 and 12 for R_3 and 9L cells, respectively. Spheroids grown from 9L cells, R_3 cells, or mixtures of both (mixed-cell spheroids) were initiated by adding single-cell suspensions of 9L and R_3 cells, obtained from exponentially growing monolayer cultures, to spinner flasks in specified ratios (*8*).

Mixed-cell spheroids with diameters of 200 to 300 μm were treated for 1 hour with 3 μ*M* BCNU, dissociated into single cells, and the SCE assay was performed (*3*). Histograms were generated by plotting the number of cells against the number of SCE's per metaphase (Fig. 1). For each mixture (Fig. 1, c to e) there are two distinct areas in the histogram corresponding to the R_3 and 9L populations, the percentages of which are very similar to the percentages of mixtures used to initiate spheroids. This procedure was repeated and histograms were generated for mixed-cell spheroids with diameters of 100 to 200 and 400 to 500 μm (Table 1); results were similar to those determined for spheroids of 200 to 300 μm. From Fig. 1 and Table 1 it is clear that the initial proportions of R_3 and 9L cells added as single-cell suspensions were maintained during spheroid formation and growth up to diameters of at least 500 μm.

Because induction of SCE's is correlated with cell kill for 9L cells treated with BCNU, a change in the number of SCE's per metaphase in 9L or R_3 cells would reflect changes in cellular sensitivity to BCNU, which are not detected readily by the cell survival assay (*3*). Comparison of regions corresponding to 9L populations in all histograms suggested that, as the percentage of R_3 cells was increased, the relative sensitivity of 9L cells to BCNU decreased. To quantitate this finding, the mean number of SCE's per metaphase for the 9L cell population in each mixture was calculated and plotted against the percentage of R_3 cells in the spheroid (Fig. 2). The results indicate that in spheroids an interaction occurs between the two cell lines that causes a decrease in the sensitivity of 9L cells to BCNU. The mean number of SCE's per metaphase in the R_3 cell population was

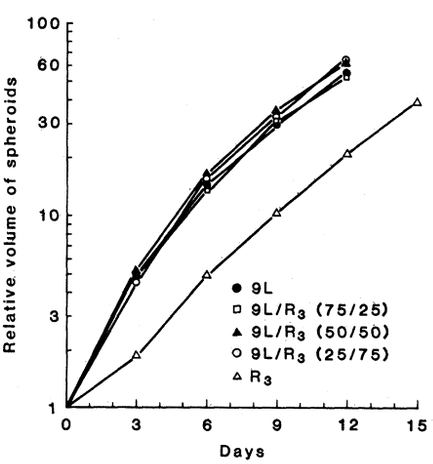

Fig. 3. Growth curves for spheroids (initial diameter, 100 to 200 μm) grown from various proportions of 9L and R_3 cells.

approximately the same in each mixture.

In studies of cell subpopulations isolated from a mouse mammary carcinoma, Heppner *et al.* (*1, 9*) found that specific subpopulations with different growth rates cultured together in monolayer grew at the rate at which one or the other subpopulation grew when cultured alone. We observed a similar effect on the growth of mixed-cell spheroids. Growth curves for untreated spheroids show that 9L spheroids grew faster than R_3 spheroids, yet mixed-cell spheroids grew at the rate of 9L spheroids (Fig. 3). Over the range of spheroid sizes and cell mixtures examined, growth rate was independent of the percentage of 9L and R_3 cells in the initial mixtures (Fig. 3). Because the percentages of 9L and R_3 cells in the initial mixtures were maintained in spheroids with diameters up to 500 μm (Table 1), results suggest that the growth of R_3 cells is increased because of an interaction with 9L cells. The interaction that affects the growth rate of tumor cell subpopulations reported here for spheroids and by others for monolayer culture (*1, 9*) may explain why the fastest growing cell type in a tumor does not become the dominant cell population and why the cellular heterogeneity of the tumor is maintained.

Cell populations of human and animal tumors are heterogeneous with respect to drug sensitivity, growth rate, and other biological characteristics (*10*). Heterogeneity is thought to be a major obstacle to successful cancer therapy. While interactions between tumor cell subpopulations may influence the generation and maintenance of heterogeneity, tumor progression, and response to therapy (*1*), their role in the biological behavior of tumors in situ remains unknown. Use of the spheroid system with the SCE assay to study interactions between cell subpopulations provides a model that in many ways simulates the tumor microenvironment. Moreover, the effects of drug treatment on individual cells can be determined quantitatively. Results obtained with this model may provide a greater understanding of the role of cell-cell interactions in tumor biology.

PHILIP J. TOFILON*
NEIL BUCKLEY
Brain Tumor Research Center of the Department of Neurological Surgery, School of Medicine, University of California, San Francisco 94143
DENNIS F. DEEN†
Brain Tumor Research Center of the Department of Neurological Surgery and *Department of Radiation Oncology, School of Medicine, University of California, San Francisco 94143*

References and Notes

1. G. H. Heppner, B. E. Miller, F. R. Miller, *Biochim. Biophys. Acta* **695**, 215 (1983).
2. R. Sutherland, J. Carlsson, R. Durand, J. Yuhas, *Cancer Res.* **41**, 2980 (1981).
3. P. J. Tofilon, M. E. Williams, D. F. Deen, *ibid.* **43**, 473 (1983).
4. P. J. Tofilon, S. M. Oredsson, D. F. Deen, L. J. Marton, *Science* **217**, 1044 (1982).
5. P. J. Tofilon and D. F. Deen, *Radiat. Res.* **97**, 171 (1984).
6. P. J. Tofilon, K. T. Wheeler, D. F. Deen, *Eur. J. Cancer Clin. Oncol.* **20**, 927 (1984).
7. M. L. Rosenblum *et al.*, in *Modern Neurosurgery*, M. Brock, Ed. (Springer-Verlag, Berlin, 1982), p. 104.
8. D. F. Deen *et al.*, *J. Natl. Cancer Inst.* **64**, 1373 (1980).
9. G. H. Heppner, in *Tumor Cell Heterogeneity: Origins and Implications*, A. H. Owens *et al.*, Eds. (Academic Press, New York, 1982), p. 225.
10. D. L. Dexter and P. Calabresi, *Biochim. Biophys. Acta* **695**, 97 (1982).
11. We thank C. Tofilon for typing the manuscript. This research was supported by NIH grants CA-09215, CA-31867, CA-31868, and CA-13525 and by the Andres Soriano Cancer Research Fund.
* Present address: Department of Experimental Radiotherapy, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030.
† To whom requests for reprints should be sent.

11 June 1984; accepted 27 July 1984

Cigarette Craving, Smoking Withdrawal, and Clonidine

Abstract. *Clonidine, an α-2-adrenergic agonist, significantly reduces opiate withdrawal. Fifteen heavy smokers abstained from cigarettes on three separate occasions and received instead clonidine, placebo, or the benzodiazepine alprazolam. Clonidine and alprazolam diminished withdrawal symptoms. The two drugs suppressed anxiety, tension, irritability, and restlessness equally but clonidine had a greater effect than alprazolam on cigarette craving. These observations suggest that noradrenergic activity is a common feature in the pathophysiology of withdrawal and that a special relationship exists between central noradrenergic activity and craving.*

The α-2-noradrenergic agonist clonidine diminishes the opiate withdrawal syndrome in chronically addicted human subjects (*1*). Central noradrenergic function had long been implicated in the action of opiates, but the anatomical locus for that interaction remained unknown. In the 1970's, evidence began to accumulate that a major anatomical connection between the adrenergic and opiate systems existed in the locus coeruleus. This nucleus accounts for nearly half of the noradrenergic neurons and produces the majority of norepinephrine in the mammalian brain. Its noradrenergic cells are densely populated with inhibitory opiate receptors. Enkephalins and opiates as well as α-2-noradrenergic agonists decrease the firing rate of these cells, and abrupt opiate withdrawal results in a marked increase in this firing rate (*2*). Extensive data have now accumulated from both experimental animals (*3*) and man (*4*) confirming Gold's original observation (*1*), and animal data support the assertion that this diminished withdrawal behavior is related to diminished noradrenergic activity (*5*).

We asked whether this concept of noradrenergic involvement in opiate withdrawal could be extended to appetitive behaviors such as smoking. We now report that clonidine alters the acute withdrawal syndrome associated with cigarette smoking and suggest that central adrenergic overactivity is a common feature in the pathophysiology of withdrawal syndromes seen with a variety of addictive substances, including cigarettes, alcohol, and opiates.

Volunteers smoking more than 30 cigarettes per day for at least 1 year, were recruited to participate in a double-blind crossover study of the effects of clonidine on the acute smoking withdrawal syndrome. In addition to a placebo control, a benzodiazepine-like drug, alprazolam, was used in a second experimental condition. Alprazolam has been shown to be equally anxiolytic and slightly less sedative than diazepam (*6*).

All subjects were in good health and were drug-free, except for two female volunteers who used medication for birth control. All subjects were instructed to refrain from smoking for 24 hours on three separate occasions. On each occasion they were told not to smoke after going to bed and to report without smoking at 0830 the next morning. Baseline pulse, blood pressure, and psychological measures were obtained; then one of three treatment regimens was begun. Subjects received clonidine (0.2 mg), alprazolam (1.0 mg), and placebo in one of three randomly assigned sequences. All treatments were given in two divided doses with the second dose given 90 minutes after the first. Pulse and blood pressure, including orthostatic blood pressure, were measured every 90 minutes. At the same time, subjects completed a series of nine visual analog scales. These scales used a 10-cm line to assess tension, anxiety, irritability, craving (thoughts about or wish to smoke), restlessness, impaired concentration, and sadness (or tearfulness). Subjects also completed similar scales for drowsiness and dizziness. At the end of each experimental day, they made a global assessment (on a scale of 1 to 10) of the