Noncycling Tumor Cells: Mitogenic Response to Antilymphocytic Serum

Abstract. *After continuous labeling with tritiated thymidine for a period several times the cell generation time, some ELD ascites cells remained unlabeled. Despite continued exposure to tritiated thymidine, unlabeled mitoses appeared promptly after administration of mouse antilymphocytic serum. Immunosuppression released some noncycling G_2 tumor cells into mitosis.*

When experimental or human tumors were continuously exposed to tritiated thymidine (^{3}H-TdR), some apparently viable tumor cells did not acquire the label (*1*). The unlabeled tumor cells have been designated by several conceptual terms with varying shades of meaning: "nonproliferating cells"; "nongrowth fraction"; "G_0 cells"; "G_1 and G_2 population cells"; "resting cells"; "nondividing subpool"; and "noncycling cells." We now report a functional basis for some of the noncycling tumor cells.

In our studies the noncycling pool has been identified in the Ehrlich ELD ascites tumor in DBA/2J mice by a combination of autoradiographic and cytophotometric techniques. After administration of ^{3}H-TdR continuously (by intraperitoneal injection every 4 hours) for several times the cell generation time of the tumor, unlabeled tumor cell nuclei are characterized by Feulgen absorption microspectrophotometry as 2C-DNA (1C refers to amount of DNA in gametes) content, or G_1-population cells, and as 4C-DNA content, or G_2-population cells (*2*).

The cell generation time of the tumor, as measured by the method of labeled mitoses, is about 16 hours with a G_2 period of 4 hours and an S (DNA synthesis) period of 7 hours. The cell generation time (or cell cycle time) should be contrasted with the doubling time of the tumor, which was determined 7 days after implantation by serial measurement of ascitic volume and counts of tumor cells and was found to be 40 hours. The ELD tumor has a hyperdiploid mode of 45 chromosomes with about 11 percent of cells at a near tetraploid peak. Mice can easily be immunized against the ELD ascites tumor and the LD_{50} (number of tumor cells that kill 50 percent of animals injected) cell transfer dose is high (*3*).

In the initial experiment five DBA/2J mice with an ELD ascites tumor were given 10 μc of ^{3}H-TdR (Schwarz BioResearch, specific activity, 1.9 c/mmole) intraperitoneally every 2 hours for 36 hours and then every 4 hours for the next 56 hours, a total of 96 hours for the experiment (*4*). The experiment was initiated on day 5 after tumor transfer. Smears of ascitic fluid were dried, fixed in a modified Carnoy's solution, hydrolyzed in 5*N* HCl at room temperature, stained with Schiff's reagent, dipped in NTB2 liquid emulsion, and autoradiographed for 8 days (*5*). The DNA contents of unlabeled nuclei were measured by a one-wavelength, two-area method (*6*).

At the end of 96 hours of continuous labeling, unlabeled interphase cells were still present, averaging 4.2 percent and ranging from 1.9 to 6.8 percent. An occasional unlabeled mitosis was observed. DNA contents were measured by cytophotometry in 100 consecutive unlabeled interphase nuclei at 96 hours and compared to respective controls at 0 time.

Frequency histograms of DNA content in tumor cell nuclei (Fig. 1) revealed the presence of unlabeled interphase nuclei in the G_1 period (2C-DNA content) and in the G_2 period (4C-DNA content). A few of the unlabeled 4C nuclei may be regarded as tetraploid tumor cells in the G_1 period. The G_2 cells that were unlabeled at the end of 96 hours of continuous labeling had been "resting" in that period from the time the experiment began, because any cycling cell would have passed through S and thereby acquired label.

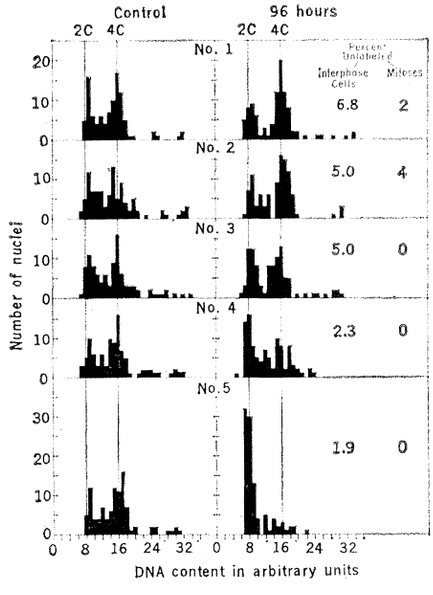

Fig. 1. DNA frequency histograms of 100 consecutive unlabeled interphase nuclei in five ELD ascites tumors before and after 96 hours of continuous labeling with ^{3}H-TdR.

The unlabeled, or noncycling, interphase tumor cells were considered members of one or more of three possible groups: (i) cells that represented the tail of a skewed distribution of cell generation times; (ii) sterile cells incapable of entry into DNA synthesis or cell division; or (iii) metabolically competent cells shunted in some way from the cell cycle.

In the studies being reported, rabbit antiserum to mouse lymphocytes (ALS) was administered intraperitoneally following 48 hours of continuous labeling. The ALS was prepared by the method of Levey and Medawar (*7*).

Twelve DBA/2J mice were implanted intraperitoneally with 125×10^6 viable Ehrlich ascites cells. On the 5th day after implantation, ^{3}H-TdR was initiated by intraperitoneal injection of 10 μc every 4 hours. At the end of 48 hours after the injection of ^{3}H-TdR, one group of six animals received 0.5 ml of ALS, intraperitoneally; the control group received 0.5 ml of saline, intraperitoneally. Colchicine, 0.1 mg in 0.1 ml, was also administered intraperitoneally to each animal of both groups. Tritiated thymidine, 10 μc, was continued at 4-hour intervals. Samples of ascitic fluid were removed at 49, 51, 54, 57, and 60 hours, at which time the experiment was terminated. Smears were dried, fixed, stained by the Feulgen method, and autoradiographed. The slides from animals in the control group and those treated with ALS were prepared in an identical manner. In each slide, 50 consecutive mitoses were classified as either labeled or unlabeled. The frequency of unlabeled mitoses at a time period was contrasted by chi-square tests in the respective control and treated groups.

At 48 hours, an average of 1.4 percent of mitoses was unlabeled (Fig. 2). Thereafter, the frequency of unlabeled mitoses declined slightly in the control (saline-treated) animals. One hour after injection of ALS (49 hours), 5.3 percent of the mitoses were unlabeled ($P < .10$). At 51 hours, 5.7 percent of mitoses were unlabeled ($P < .05$);

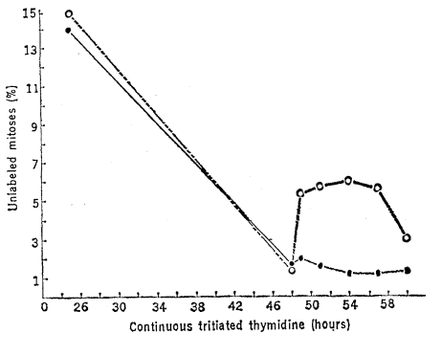

Fig. 2. Percentage of unlabeled mitoses during continuous labeling with ^{3}H-TdR, in controls (solid circles) and following administration of antiserum to mouse lymphocytes (open circles) after the 48-hour sample.

at 54 hours, 6.0 percent ($P < .01$); at 57 hours, 5.7 percent ($P < .01$); and at 60 hours, 3.0 percent were unlabeled ($P < .25$). One of the six tumors treated with ALS showed no response; the means above, however, include all six animals. In the later time periods, anaphases and telophases appeared following release of inhibition by colchicine in both groups.

At 48 hours the percentage of unlabeled interphase cells averaged 22.0 percent. Subsequently, the percentage of unlabeled interphase cells did not differ significantly in the two groups. The mitotic index increased in both groups after colchicine was given at 48 hours. The increase in the mitotic index was slightly, but insignificantly, greater in the group treated with ALS. Cytophotometry of unlabeled interphase tumor nuclei, contrasting the 48- and 54-hour samples in each animal, showed a modest but insignificant depletion of 4C, or G_2, nuclei in the 54-hour smears of the tumors treated with ALS.

The peripheral white blood cell count of mice with a 7-day-old ELD ascites tumor averaged 19,600 cells per cubic millimeter. Four hours after administration of ALS, the peripheral white blood cell count averaged 14,000 per cubic millimeter. A lymphopenia was observed in the intraperitoneal fluid after injection of ALS.

With immunosuppression, some unlabeled tumor cells were released promptly into mitosis, entering the cycling pool. Cytophotometry showed that there were many more unlabeled G_2 cells than could be stimulated into mitosis during the experiment. Our previous studies showed a similar mitogenic effect by hydrocortisone at 48 hours and at 96 hours of continuous labeling (*8*) and by azathioprine at 48 hours labeling. The presence or absence of colchicine made no difference. There was no mitogenic effect from distilled water or isotonic saline.

We conclude that some noncycling tumor cells were probably restrained by immune inhibition. The mitogenic response to ALS occurred within 4 hours after injection, persisted for about 6 hours, and then gradually subsided. Since unlabeled mitoses appeared promptly after treatment with ALS, despite continuing administration of ^{3}H-TdR, and since some unlabeled dividing tumor cells escaped the colchicine block to finish mitosis, we conclude further that immune restraint was probably exerted on metabolically competent, DNA-replicated tumor cells in the G_2 period of interphase. Immune inhibition is therefore one of the parameters controlling tumor growth in this experimental system (*9*). These results have a bearing on the concept of the dormant tumor cell.

Immunological control mechanisms have been demonstrated in experimental tumors by a variety of methods (*10*). Allogeneic and isogeneic lymphocytes have depressed tumor growth, possibly by inhibition of DNA synthesis (*11*). Antilymphocytic serum has increased the growth rate of tumors in mice (*12*) but had an unpredictable effect in another study (*13*).

The effect of ALS is apparently mediated by host lymphocytes (*14*). Action of sensitized lymphocytes is directed toward alteration of antigens bound to the surface of the tumor cell (*15*). Our evidence then suggests that administration of ALS, acting via immunologically competent host lymphocytes, resulted in a cell surface event that initiated mitosis in noncycling, G_2 population, ascites tumor cells.

JEROME J. DECOSSE
Department of Surgery, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

SEYMOUR GELFANT
Department of Zoology, Syracuse University, Syracuse, New York 13210

References and Notes

1. M. L. Mendelsohn, *Science* **135**, 213 (1962); B. Clarkson, K. Ota, T. Ohkita, A. O'Connor, *Cancer Res.* **18**, 1189 (1965); J. J. DeCosse and S. Gelfant, *Proc. Amer. Ass. Cancer Res.* **7**, 17 (1966); M. Shiuming Lin and B. Bouroncle, *Blood* **29**, 63 (1967).
2. S. Gelfant, in *Methods in Cell Physiology*, D. M. Prescott, Ed. (Academic Press, New York, 1966), vol. 2, p. 359.
3. T. S. Hauschka, S. T. Grinnell, L. Revesz, G. Klein, *J. Nat. Cancer Inst.* **19**, 13 (1957).
4. J. J. DeCosse and S. Gelfant, *Proc. Amer. Ass. Cancer Res.* **7**, 17 (1966).
5. J. J. DeCosse and N. Aiello, *J. Histochem. Cytochem.* **14**, 601 (1966).
6. A. M. Garcia, *ibid.* **13**, 161 (1965).
7. R. H. Levey and P. B. Medawar, *Proc. Nat. Acad. Sci. U.S.* **56**, 1130 (1966).
8. J. J. DeCosse and S. Gelfant, *Surg. Forum* **18**, 80 (1967).
9. P. K. Lala and H. M. Patt, *Proc. Nat. Acad. Sci. U.S.* **56**, 1735 (1966).
10. E. J. Foley, *Cancer Res.* **13**, 835 (1953); M. H. Flax, *ibid.* **16**, 774 (1956); B. T. Prehn, *J. Nat. Cancer Inst.* **31**, 791 (1963); K. E. Hellström, I. Hellström, G. Haughton, *Nature* **204**, 661 (1964); M. Brenneman and P. G. Rigby, *Brit. J. Cancer* **22**, 58 (1968); G. Klein, *Cancer Res.* **28**, 625 (1968); R. T. Smith, *New Engl. J. Med.* **278**, 1207 (1968).
11. G. Klein and E. Klein, *Cold Spring Harbor Symp. Quant. Biol.* **27**, 463 (1962); E. Möller, *Science* **147**, 873 (1965); S. Ming, E. Klein, G. Klein, *Nature* **215**, 1390 (1967).
12. B. Phillips and J. C. Gazet, *Nature* **215**, 548 (1967); S. D. Deodhar, G. Crile, Jr., P. F. Schofield, *Lancet* **1968-I**, 168 (1968).
13. S. Bremberg, E. Klein, J. Stjernswärd, *Cancer Res.* **27**, 2113 (1967).
14. R. H. Levey and P. B. Medawar, in *Antilymphocytic Serum*, G. E. W. Wolstenholme, Ed. (Little, Brown, Boston, 1967), p. 272.
15. D. F. H. Wallach and E. B. Hager, *Nature* **196**, 1004 (1962); K. E. Hellström and I. Hellström, *Progr. Exp. Tumor Res.* **9**, 40 (1967).
16. Supported by NIH grant CA 10381 and NSF grant GB-2803. J.J.D. is a Markle scholar in academic medicine. We thank Dr. H. DiStefano for valuable advice, Dr. K. S. Warren for provision of the antilymphocytic serum, Miss K. Dugan for technical support, and Dr. L. McCorkle for statistical assistance. A portion of this work was performed in the Department of Anatomy, State University of New York, Upstate Medical Center, Syracuse.

24 June 1968; revised 9 September 1968

RNA Competition in RNA-DNA Hybridization Systems

It is interesting that results of Riggsby and Merriam (*1*) are explained by the findings of Britten and Kohne in the same issue of *Science* (*2*). Riggsby and Merriam found that in mammalian systems there is competition between RNA species in RNA-DNA hybridization systems only if the ratio of RNA to DNA is relatively high. The observations by Britten and Kohne that there are many hundreds of thousands of repeated sequences in DNA of higher animals readily explain why a high RNA/DNA ratio is needed. If the RNA/DNA ratio is low there will still be many of the redundant DNA sites to be covered by the RNA. In bacteria there is no redundancy of DNA sequences, hence this phenomenon is not observed.

JAY S. ROTH
Biochemistry and Biophysics Section, University of Connecticut, Storrs 06268

References

1. W. S. Riggsby and V. Merriam, *Science* **161**, 570 (1968).
2. R. J. Britten and D. E. Kohne, *ibid.*, p. 529.

16 September 1968