Formation of Mast Cell Granules in Cell Cycle Mutants of an Undifferentiated Mastocytoma Line: Evidence for Two Different States of Reversible Proliferative Quiescence

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ABSTRACT A heat-sensitive (hs, arrested at 39.5°C, multiplying at 33°C) and a cold-sensitive (cs, arrested at 33°C, multiplying at 39.5°C) cell cycle variant were isolated from an undifferentiated P-815 murine mastocytoma line. At the respective nonpermissive temperature, both the hs and the cs variant cells were reversibly arrested with a DNA content, typical of G1 phase. The cells of two cs variant subclones, when exposed to the nonpermissive temperature of 33°C, formed metachromatically staining granules with an ultrastructure resembling that of mature mast cells. In addition, the cellular 5-hydroxytryptamine content underwent a marked increase, and the cells responded to compound 48/80 by degranulation as described for normal mast cells. On the other hand, in cells of two hs variant subclones, essentially no mast cell granules were detectable at either 33 or 39.5°C. As previously reported, the cs cell cycle variant phenotype is expressed dominantly in heterokaryons obtained by fusing cs with wild-type cells, whereas hs cell cycle variant cells, similar to other hs mutants, were found to behave recessively under these conditions. Thus the state of proliferative quiescence induced in the cs cells at 33°C is qualitatively different from the state of cell cycle arrest observed in hs cells at 39.5°C and may represent a model for proliferative quiescence of differentiated cells in the intact organism.

Conditional cell cycle mutants of animal cells represent promising tools for analyzing mechanisms involved in the transition of a cell from proliferative activity into a state of reversible proliferative quiescence (commonly referred to as G0 phase) and back into active cell proliferation. Whereas heat-sensitive (hs, arrested at ~39.5°C, multiplying at ~33°C) cell cycle mutants are presently available in relatively large numbers, only a few cold-sensitive (cs, arrested at 33°C, multiplying at 39.5°C) cell cycle mutants are presently available in relatively large numbers, only a few cold-sensitive (cs, arrested at 33°C, multiplying at 39.5°C) cell cycle mutants have been described. We have recently reported that a cs cell cycle mutant of the murine P-815 line exhibits dominant behavior in heterokaryons obtained by fusion with wild-type cells (1), whereas hs mutants generally behave recessively (1, 2). Since the cells of the P-815 line and the P-815-derived cs and hs cell cycle mutants have been described. We have recently reported that a cs cell cycle mutant of the murine P-815 line exhibits dominant behavior in heterokaryons obtained by fusion with wild-type cells (1), whereas hs mutants generally behave recessively (1, 2). Since the cells of the P-815 line and the P-815-derived cs and hs cell cycle mutants grow in suspension without attaching to solid substrates, cell-to-cell interactions such as contact inhibition may be minimized in studying the effects of various culture conditions. This communication shows that cs cell cycle mutants also differ from hs cell cycle mutants with respect to their capacity to undergo cellular differentiation when brought to the nonpermissive temperature.

MATERIALS AND METHODS

Cell Lines and Culture Techniques: A clonal subline (K 21) of the P-815-X2 mouse mastocytoma, the cells of which multiply in suspension without attaching to a solid substrate (3–5), was used for selecting an hs and a cs variant as described by Zimmermann et al. (1). Clonal sublines were derived by isolating single cells from the two variant cell lines. Characteristics of two subclones of the hs line (21-Tb and 21-Td), and two subclones of the cs line (21-Fb and 21-Fc) were compared with those of the parent clone (K 21) and are presented in this communication. Unlike early passage P-815 cells (3), the P-815-X2 as well as the K 21 cells used for selection of the variants contained essentially no metachromatically staining granules. A culture medium referred to as medium...
I (6) and supplemented with 10% horse serum was used. Cell multiplication was measured by determining the cell density (cell number per milliliter) with a Coulter counter (Coulter Electronics, Hialeah, FL). The cultures were diluted every 24 h with fresh medium to obtain 200,000 cells/ml. If cell density after 24 h of incubation was <267,000/ml, part of the cells was centrifuged and resuspended in fresh medium to supply the culture with at least 25% of fresh medium per day. To determine relative numbers of colony-forming cells, the cells were cultured after appropriate dilution with medium in fibrin gels (7), and colonies that developed at the respective permissive temperature were counted after 1–4 wk of incubation.

**Autoradiography:** DNA-synthesizing cells were labeled by incubating aliquots of cultures with [3H]thymidine at a concentration of 0.5 μCi/ml. For autoradiography, the cells were incubated for 10 min at 37°C in 70 mM KCl fixed by adding 9 vol of a cold mixture of ethanol and acetic acid (10:1), resuspended in 0.08 ml of this mixture, stored at −20°C for 15 min, and brought onto glass slides. The dried preparations were covered with Kodak NTB-2 emulsion, exposed for 1–2 wk, developed, and stained with Giemsa’s. Cell nuclei were considered to be labeled if covered by more than 15 silver grains. Labeling indices were determined by evaluating 500-1000 cells per preparation.

**Morphological Evaluation:** For light microscopy, the cells were incubated for 10 min at 37°C in 70 mM KCl, fixed, and brought onto glass slides as described above. The dried preparations were stained with toluidine blue (Ciba-Geigy Corp., Basel; 1% solution in water) for 30 min and examined for cytoplasmic granules with typical metachromasia. In each preparation, numbers of metachromatic granules were determined in at least 300 cells. To test the capacity of granule-containing cells to respond to a degranulating agent (8), compound 48/80 (the condensation product of N-methyl-p-methoxyphenethylamine with formaldehyde; Sigma Chemical Co., St. Louis, MO) was added to cultures of 21-Fb and 21-Fc subclones at a final concentration of 1 μg/ml.

In preparation for electron microscopy, the cells were centrifuged to obtain a pellet, fixed with glutaraldehyde (2.5% in 0.14 mM cacodylate buffer), postfixed with osmium tetroxide, dehydrated with graded acetone, embedded in Spurr’s low viscosity medium (Taab Laboratories, Reading, England), and stained with uranyl acetate and lead citrate. Ultrathin sections were examined in a Zeiss EM 10 transmission electron microscope.

**Determination of Cellular 5-Hydroxytryptamine Content:** Approximately 10^7 cells were centrifuged, resuspended in 2 ml of isotonic NaCl, mixed with 2 ml of 0.8 N HClO4, and heated for 10 min at 80°C. After centrifugation, 5-hydroxytryptamine in the supernatant was extracted and assayed according to the method of Snyder et al. (9).

**RESULTS**

**Cell Multiplication and Formation of Mast Cell Granules**

Multiplication of hs 21-Tb and 21-Tf cells, cs 21-Fb and 21-Fc cells, and K 21 cells at 33 and 39.5°C is presented in Fig. 1 (top). K 21 cells exhibited exponential proliferation at both temperatures. Multiplication of hs 21-Tb and 21-Tf cells nearly ceased after the first 24 h at 39.5°C, whereas after incubating cs 21-Fb and 21-Fc cells at 33°C for 3 d, almost no further increase in cell number was detected. In contrast, the cell multiplication rate of hs 21-Tb and 21-Tf cells at 33°C and of cs 21-Fb and 21-Fc cells at 39.5°C was similar to that of K 21 cells. As previously described for the 21-Ta and the 21-Fb clone, after shift of hs and cs cells to the respective nonpermissive temperature, a progressive accumulation of cells with a DNA content, typical of G phase, was observed (1).

When cells of cs 21-Fb and 21-Fc clones were incubated at the nonpermissive temperature of 39.5°C, formation of large numbers of metachromatically staining cytoplasmic granules was observed (Fig. 1, bottom). During exponential multiplication of these cs cells at 39.5°C, however, essentially no granules were detected. As exemplified in Fig. 2, >97% of 21-Fb cells maintained at 39.5°C contained no detectable metachromatically staining granules at all, whereas after 6 d at 33°C, >94% of the cells contained at least 10 granules, with a relatively broad distribution of granule number per cell. In contrast with the cs subclones, nearly no metachromatically staining granules were present in hs 21-Tb and 21-Tf cells that were incubated at the nonpermissive temperature of 39.5°C for up to 6 d. Similarly, 21-Tb and 21-Tf cells multiplying exponentially at 33°C, as well as K 21 cells at both 33°C and 39.5°C, contained essentially no metachromatic granules. As an additional control, a serum-dependent variant (21-SA3; multiplying in medium with 10% horse serum, arrested within 3 d in serum-free medium containing 0.2% bovine serum albumin) was derived from K 21 cells. When 21-SA3 cells were cultured at 33°C in serum-free medium containing BSA for up to 6 d, essentially no metachromatically staining granules were detectable.

To further characterize the functional change that occurred...
during incubation of cs cells at 33°C, the 5-hydroxytryptamine (5-HT) content of 21-Fb cells was determined. In exponentially multiplying cells maintained at 39.5°C, the average 5-HT content thus obtained was 2 ± 0.2 ng/10^7 cells, whereas after 6 d at 33°C, the cellular 5-HT content had increased to 38 ± 3 ng/10^7 cells (mean ± SE from three independent experiments).

Electron microscopy of cs 21-Fb cells that had been incubated for 6 d at 33°C revealed granules with an ultrastructural morphology typical of mast cell granules in different phases of maturation (Fig. 3, right). On the other hand, essentially no mature granules were detectable in electron micrographs of cs 21-Fb cells multiplying exponentially at 39.5°C (Fig. 3, left), of hs 21-Tb and 21-Tf cells arrested at 39.5°C or proliferating at 33°C, and of K 21 cells at both temperatures.

**FIGURE 2** Distribution of granule number per cell in a population of cs 21-Fb cells. (A) 21-Fb cells maintained at the permissive temperature of 39.5°C; (B) 21-Fb cells after 6 d of incubation at 33°C.

**FIGURE 3** Electron micrographs of cs 21-Fb cells during incubation at the permissive temperature of 39.5°C (left; × 1.8 × 10^3), and after 6 d of incubation at the nonpermissive temperature of 33°C (right; × 1.5 × 10^3). Whereas the cytoplasm of the cell incubated at 39.5°C contains no mature mast cell granules, the cells that were incubated at 33°C contain numerous typical mast cell granules. Inset: Mature granule of a 21-Fb cell at × 2.0 × 10^4.

Cells of cs 21-Fb and 21-Fc clones, after incubating at 33°C for 6 d, were exposed to compound 48/80. Under these conditions, the granule-containing cells underwent progressive degranulation (Fig. 4).

**Reversibility of Proliferative Quiescence**

The arrest of cell multiplication of cs 21-Fb and 21-Fc and of hs 21-Tb and 21-Tf cells at the respective nonpermissive temperature was found to be reversible, i.e., DNA synthesis and cell multiplication were resumed if cells were brought back to the permissive temperature. This is exemplified in Fig. 5 for cs 21-Fc cells that were incubated at 33°C for up to 6 d before reincubation at the permissive temperature of 39.5°C. We observed, following 2, 4, and 6 d of incubation at 33°C that a marked increase in the number of cells entering the S period occurred between 16 and 24 h after return to 39.5°C; at 48 h after reincubation at 39.5°C, relative numbers of DNA-synthesizing cells were comparable to, or even somewhat higher than, those of control cultures not exposed to 33°C.

To test whether proliferative quiescence of cs cell cycle mutants was reversible, irrespective of the degree of cellular differentiation, 21-Fb cells were arrested by incubation at 33°C. After 6 d, [³H]thymidine was added, the cells were brought back to 39.5°C, and at various times, aliquots of the cell suspension were processed for autoradiography and toluidine blue staining. Under these conditions, essentially the same numbers of mast cell granules were observed in labeled, as compared with unlabeled, cells, indicating that the capacity to reenter the cell cycle was not restricted to cells with a low degree of cell differentiation. For instance, at 48 h after return of the cells to 39.5°C, when 57 ± 2.5% of the cells were [³H]-labeled, the mean number of mast cell granules per labeled cell was 13.9 ± 0.3, whereas the corresponding number for unlabeled cells was 12.3 ± 0.2 (means ± SE of three independent experiments). It should be noted that in these experiments the numbers of observed granules per cell were somewhat lower due to the limited visibility of small granules caused by the autoradiographic emulsion. During the incubation of 21-Fb cells at 39.5°C, the mean number of granules per cell gradually decreased, and after 9 d, no mast cell granule was detectable anymore in the majority of the cells. The time course of this
decrease is compatible with the assumption that at 39.5°C essentially no new granules were formed, whereas resuming exponential cell multiplication resulted in the repeated distribution of preexisting granules between daughter cells.

As an additional test of the reversibility of proliferative quiescence at the respective nonpermissive temperature, the capacity of cell cycle mutants to form colonies at the permissive temperature after different incubation periods at the nonpermissive temperature was determined. The results presented in Table I indicate that relative numbers of colony-forming cells remained essentially constant during culture of cs 21-F cells at 33°C, hs 21-T cells at 39.5°C, and K 21 cells at both temperatures for up to 7 d. As seen in Table I, colony yields obtained by cloning on day 0 tended to be somewhat higher than on subsequent days, suggesting that changing the incubation temperature at the time of cloning, as applied on days 1-7, may have adversely affected some of the cells.

**DISCUSSION**

As previously discussed (1), the results of cell fusion experiments support the conclusion that hs cell cycle variants of the P-815 line contain a heat-labile gene product that is required for traverse through G1 phase and that is inactive at the nonpermissive temperature of 39.5°C. On the other hand, the dominant expression of the cs phenotype of 21-Fb cells, i.e., the capacity to prevent wild-type nuclei in heterokaryons from entering S phase, suggests that at the nonpermissive temperature of 33°C, a gene product is formed in the cs cells that induces a state of reversible proliferative quiescence. At the permissive temperature of 39.5°C, this effect is not observed, presumably because the gene product responsible for inducing proliferative quiescence is inactivated.

The results presented in this communication demonstrate that the hs 21-T cell cycle variants also differ from the cs 21-F cell cycle variants with respect to their capacity to undergo cellular differentiation when brought to the respective nonpermissive temperature. Whereas in the hs variants arrested at 39.5°C essentially no metachromatic granules were formed, the cs variants, after the temperature shift to 33°C, exhibited a pronounced increase in the number of granules. In addition to the metachromatic staining properties of the granules, the observed increase in 5-hydroxytryptamine content of 21-Fb cells after the shift to 33°C supports the conclusion that these granules, even though morphologically they represent varying degrees of maturation, are biochemically similar to granules of normal murine mast cells. Furthermore, the cs 21-F cells, after being arrested at 33°C, responded to compound 48/80 by degranulation, thus expressing another complex function of normal mast cells (8). The cs 21-F cells are, therefore, reminiscent of a cs cell cycle mutant of CHO cells with features of reverse transformation at the nonpermissive temperature of 33°C (10). The granules formed in cs 21-Fb and 21-Fc cells at 33°C appear comparable in number and appearance to those that are formed in a clonal subline (P-815-4) of P-815 cells during incubation with sodium butyrate (11). On the other hand, absolute amounts of 5-hydroxytryptamine in 21-Fb cells maintained at 39.5°C or 33°C were lower than those reported for P-815-4 cells (12), and unlike P-815-4 cells treated with butyrate, the 21-Fb and 21-Fc cells had no tendency to adhere to the glass surface of culture bottles at 33°C or 39.5°C.

The results obtained are compatible with the assumption that the gene product responsible for the cs phenotype of 21-Fb and 21-Fc cells may be a pleiotropic effector, inducing both a state a reversible proliferative quiescence and the formation formation of mast cell granules.
of mast cell granules during incubation at 33°C. At 39.5°C this
gene product would be inactive. This effector appears compar-
table to a factor in quiescent human diploid cells that, when
fused to replicative cells of the same cell line, were shown to
exert an inhibitory effect on the entry into S phase of both
nuclei (13). On the other hand, lack of activity of a gene
product required for traverse through G1 phase, as exemplified
by the hs cell cycle mutants 21-Tb and 21-Tf at 39.5°C,
apparently is insufficient for inducing mast cell differentiation.

As shown by the capacity of arrested cells to reenter the S
period and to form colonies when brought back to the permis-
sive temperature, at least a major portion of the cells of both
the cs 21-F and the hs 21-T cell cycle mutant clones enter a
state of reversible proliferative quiescence during incubation at
the respective nonpermissive temperature. Thus, cell cycle
arrest due to exposure to the nonpermissive temperature did
not result in a progressive loss of the capacity of cells to reenter
the cell cycle.

As seen in Fig. 1, during incubation of cs 21-Fb and 21-Fc
cells at 33°C, the main increase in the number of mast cell
granules occurred when cell multiplication already had nearly
ceased. Thus, the cells may first have entered a state of revers-
ible proliferative quiescence similar to the G0 state that is
attained by cells of a proadipocyte line under conditions sub-
sequently inducing adipocyte differentiation (14), whereas quie-
scence of cs 21-F cells after formation of mast cell granules
may possibly correspond to the state of nonterminal differen-
tiation described for the proadipocyte line (15). It will, there-
fore, be interesting to test using cell fusion experiments whether
cells of the proadipocyte line in the G0 state also contain a
factor preventing entry into S phase, resulting in dominant
expression of the arrested state in heterokaryons.

In conclusion, it may be appropriate to distinguish among
different states of reversible arrest of cell cycle progression.
The term "G0 phase" has previously been applied either to all
cells with a G0 content of DNA that have reversibly ceased
rapid proliferation (16, 17), or to cells that fulfill certain quan-
titative criteria, such as a long time interval between proliferative stimulation and entry into S phase. On the other
hand, the cs 21-Fb and 21-Fc cells, when incubated at the
nonpermissive temperature of 33°C, enter a G0 phase that is
qualitatively different from the G0 phase of arrested 21-Tb and
21-Tf cells. The state of proliferative quiescence induced in 21-
Fb and 21-Fc cells at 33°C, characterized by (a) dominant
expression in heterokaryons and (b) pronounced cellular dif-
ferrntiation, may represent a valuable model system because
mechanisms similar to those that are responsible for inducing
proliferative quiescence and cell differentiation of cs 21-F cells
at 33°C may be operative also in certain types of normal cells
ceasing proliferation and undergoing cell differentiation. It
will, therefore, be interesting to test whether this particular
type of proliferative quiescence can be observed also in other,
including normal, nontransformed cell types.

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