Coinfection with Viruses Carrying the *v-Ha-ras* and *v-myc* Oncogenes Leads to Growth Factor Independence by an Indirect Mechanism

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The concomitant expression of certain oncogenes can transform normal diploid rodent cells into transplantable tumorigenic cells. The mechanism by which these oncogenes collaborate is unclear. Recent findings (M. Oshimura, T. M. Gilmer, and J. C. Barrett, *Nature [London]* 316:636-639, 1985) raise the possibility that karyotypic changes, including monosomy for chromosome 15, are required to induce tumorigenicity in Syrian hamster embryo cells transfected in vitro with *v-Ha-ras* and *v-myc* DNAs. We studied the effect of the oncogenes *v-Ha-ras* and *v-myc*, introduced by viral infection, on murine hematopoietic cells. The induction of growth factor independence by the two oncogenes was used as an in vitro correlate of tumorigenicity. After a period of reduced growth rate reminiscent of the growth rate of cells in crisis, the doubly infected cells became growth factor independent. These cells showed a great variability in their karyotypes.

The in vitro culture system used was murine nonadherent, spleen-derived cells which were cultured in the presence of WEHI-3 cell-conditioned medium (WEHI-CM) (8). After 3 weeks in culture, a WEHI-CM-dependent population, consisting mainly of mast cells (Fig. 1a), reached a steady state of growth. The WEHI-CM-dependent population was maintained for several months without a significant change in cell number because of an equilibrium between proliferating, terminally differentiating, and dying cells. The growth factor provided to this population by WEHI-CM is most likely interleukin-3 (10). The responsiveness of this population to purified interleukin-3 has not yet been tested.

After 3 weeks in culture, WEHI-CM-dependent cells (2×10^6 per sample) were infected: (i) with Ha-MSV carrying the *v-Ha-ras* oncogene; (ii) with a murine recombinant retrovirus (murine myelocytomatosis virus [MMCV]) carrying the *v-myc*^{OK10} oncogene (13); and (iii) with both viruses simultaneously. (Moloney murine leukemia virus was used as the helper virus for Ha-MSV and MMCV for this experiment.) The infected cells were grown in the presence of 4% WEHI-CM. The appearance of growth factor-independent cells was assayed at 7- to 10-day intervals by determining the proportion of cells forming colonies after 10^1 to 10^4 infected cells were seeded per 6-cm dish in methylcellulose growth medium to which no WEHI-CM had been added. The number of colonies reflected the number of cells in the population able to grow autonomously in large culture volumes.

The population of mast cells in cultures infected with Harvey murine sarcoma virus (Ha-MSV) (Fig. 1b) doubled every 4 to 5 days for more than 6 months. During this time, the cells remained dependent on WEHI-CM. The effect of Ha-MSV on spleen-derived WEHI-CM-dependent mast cells has been described previously (10).

In cultures infected with the murine virus carrying the *v-myc*^{OK10} oncogene, the most striking feature was the high incidence of cell death. The percentage of trypan-blue-staining cells in the cultures varied between 50 and 70%, and

this persisted for at least 3 months of culturing in the presence of WEHI-CM. This high proportion of dead cells differed significantly from that (5 to 20%) in uninfected or Ha-MSV-infected cultures. Within 2 months, the population of mast cells in the MMCV-infected cultures was replaced by immature cells of the macrophage lineage with morphological characteristics of monocytes (Fig. 1c), the cell lineage commonly transformed by *v-myc*^{OK10} virus. The selective action of a *raf/myc* recombinant murine retrovirus on bone marrow cells, yielding transformed macrophages, was recently described by Blasi et al. (1).

Cultures infected simultaneously with both *v-Ha-ras*- and *v-myc*-containing viruses showed the same high proportion (50 to 80%) of dead cells as did cells infected only with *v-myc* virus. In contrast with the cultures infected only with *v-myc* virus, in which the number of living cells remained practically unchanged for several months, the living cell population of doubly infected cultures doubled every 2 days. The rapidly proliferating cells in the doubly infected cultures were also monocytes (Fig. 1d). They replaced the mast cells within 2 to 3 weeks.

The high incidence of cell death in the doubly infected cultures remained unchanged for 3 months of culturing in the presence of WEHI-CM. A similarly high incidence of cell death was observed in 20 clonal populations of infected cells isolated from methylcellulose medium supplemented with WEHI-CM. The continuous release of dead cells was therefore considered an inherited property of the infected cells.

Cells from the singly and doubly infected cultures were tested for their ability to form colonies in methylcellulose growth medium to which no WEHI-CM had been added. During the first 3 months, neither the singly nor the doubly infected cells gave rise to macroscopically visible colonies in repeated tests in which 10^4 cells were seeded in 6-cm culture dishes.

However, 3 months after infection, a number of significant changes were observed in the doubly infected cultures. The incidence of cell death increased to 90 to 95%, and the net growth in the cultures decreased significantly. At the same

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FIG. 1. Spleen-derived hematopoietic cells stained with Wright stain. Panels: a, uninfected mast cells; b, mast cells infected with Ha-MSV, carrying the *v-Ha-ras* oncogene; c, monocytes infected with MMCV, carrying the *v-myc*^{OK10} oncogene; d, monocytes doubly infected with Ha-MSV and MMCV; e, doubly infected, growth factor-independent cells from colony formed in methylcellulose in the absence of WEHI-CM. The cells are more differentiated than the parental monocytes: a small proportion of the cells adhered to the culture dish, and the cells reacted with a monoclonal anti-Mac-1 antibody. Panel f, Heterogeneity of cell size of doubly infected monocytes after crisis. In all instances, spleen cells from 4- to 8-week-old inbred NIH Swiss mice were cultured for 3 weeks in the presence of 4% WEHI-CM. Nonadherent cells were transferred every 4 to 5 days. After 3 weeks in culture, samples (2×10^6 nonadherent cells) were infected with 0.5 ml of filtered culture medium containing: (i) 4×10^5 focus-forming units of Ha-MSV; (ii) 2×10^5 focus-forming units of MMCV; or (iii) 4×10^5 focus-forming units of Ha-MSV plus 2×10^5 focus-forming units of MMCV. The virus inocula both contained 10^7 XC-PFU of Moloney murine leukemia virus as helper virus. The infected cells were subcultured with WEHI-CM at a concentration of 3×10^5 to 5×10^5 /ml. Number of weeks after infection: a-c, 9; d, 12; e, 39; f, 18.

time, the cells formed many large cell clusters in liquid medium. After 1 to 3 weeks, rapidly growing cells with a doubling time of 12 to 15 h emerged. These cells were very heterogeneous in size (Fig. 1f). The changes in the cultures were reminiscent of the changes observed when normal cells with a limited life-span go into crisis and cells with an unlimited growth potential emerge. Most important, when 10 or 100 cells were seeded in methylcellulose medium without WEHI-CM, 30 to 50% of this new population of cells formed large colonies. The cells in the colonies (Fig. 1e) were more differentiated than were the parental monocytes (Fig. 1f).

This acquisition of growth factor independence can be explained in two ways. A small number of doubly infected growth factor-independent cells (less than 1 of 1×10^4 to 4×10^4 cells) arose early after infection but were not detected. If the growth factor-independent cells had a higher growth rate, they would be selected for and observed at later passages. Alternatively, infection with the *v-Ha-ras*- and *v-myc*-containing viruses did not lead directly to growth factor independence but promoted genetic changes which led to growth factor independence. To decide between these two possibilities, we cloned cells from double infected

cultures in WEHI-CM-containing methylcellulose medium 5 weeks after infection, i.e., 2 months before changes were observed in the mass cultures. The clonal isolates were monocytes which were very similar to the cells of mass cultures. After 2 to 3 months of culturing, all 11 clonal isolates studied underwent changes similar to those described for the mass cultures: formation of large cell clusters, appearance of cells of heterogeneous sizes, and the appearance of cells able to form colonies in methylcellulose medium not supplemented with WEHI-CM. The appearance of growth factor-independent cells in all 11 clonal isolates after a lag of 2 to 3 months shows the late change from dependence to independence and suggests that no growth factor-independent cells preexisted in the mass cultures.

The expression of the *v-Ha-ras* and *v-myc* oncogenes, both in singly and in doubly infected cells before and after crisis, was ascertained by immunoprecipitation (Fig. 2a and b) and by Northern blotting (data not shown). The significance of the difference (Fig. 2) between the levels of expression of p21 in cells before and after crisis is being investigated.

To determine whether the growth factor-independent cells

FIG. 2. Analysis of proteins from cells infected with v-Ha-ras- and v-myc^{OK10}-containing viruses. Panel a, Immunoprecipitation of p57^{v-mycOK10} from [³⁵S]methionine-labeled cell extracts with an anti-Hu-myc-specific serum (14). Lanes: 1 and 2, cells infected with Ha-MSV, 9 months postinfection (p.i.); 3, with MMCV, 10 weeks p.i.; 4, with Ha-MSV plus MMCV, 10 weeks p.i.; 5 and 6, 6 weeks after isolation from methylcellulose in the absence of WEHI-CM. Panel b, Immunoprecipitation of p21^{v-Ha-ras} from [³⁵S]methionine-labeled cell extracts with monoclonal antibody to p21 (4). Lanes: 1, cells infected with MMCV, 8 weeks p.i.; 2, with Ha-MSV, 38 days p.i.; 3 and 4, with Ha-MSV plus MMCV, 38 days p.i.; 5 and 6, 4 weeks after isolation from methylcellulose in the absence of WEHI-CM. For panel a, 1×10^6 to 2.5×10^6 cells were labeled with 200 μ Ci of [³⁵S]methionine for 30 min. Cell lysates were heat denatured for 1 min under reducing conditions as described by Curran et al. (3), reacted with antiserum, and analyzed on a 15% sodium dodecyl sulfate-polyacrylamide gel. Lysates were adjusted to equal amounts of incorporated label. For panel b, cells were labeled for 3 h. Cell lysates were immunoprecipitated as described by Sefton et al. (11) and analyzed on a 17.5% sodium dodecyl sulfate-polyacrylamide gel. The numbers to the left of the panels indicate the position of mass markers (in kilodaltons).

showed gross karyotypic changes, an analysis of the karyotypes of singly and doubly infected cells was performed at various times after infection. In cultures infected with Ha-MSV or v-myc virus alone and analyzed over a period of 4 months, 2% (1/54) or 8% (4/52) of the cells had 39

FIG. 3. Chromosome distribution in doubly infected monocytes at various times postinfection (p.i.). Panels: a-c, before crisis; d, after crisis; e and f, cells from growth factor-independent colonies formed in methylcellulose in the absence of WEHI-CM.

chromosomes whereas 98 or 92% had 40 chromosomes, respectively. In contrast, 27% (30/111) of the doubly infected cells had hypodiploid (34-39) chromosome numbers during the first 2 to 3 months after infection (Fig. 3a to c). At the time of gross morphological changes in these cultures, the cells had karyotypes with chromosome distributions ranging from near tetraploid to near octoploid numbers (Fig. 3d). The karyotypes of cells from individual colonies, isolated from methylcellulose medium to which no WEHI-CM had been added, varied greatly within each colony and from colony to colony: cells from some colonies had chromosome numbers which varied from near-haploid to near-tetraploid numbers (Fig. 3e and 4), whereas other colonies had karyotypes near the triploid and tetraploid range (Fig. 3f). A great variability of the karyotypes within a clonal population is a frequently observed characteristic of highly tumorigenic

FIG. 4. Chromosome spreads from two cells of colony 4-A2. Panels: a, near-triploid cell with 64 chromosome; b, near-haploid cell with 19 acrocentric and 2 metacentric chromosomes.

cells and also often found in established tissue culture lines. In summary, the results show that the growth factor-independent cells had significantly changed karyotypes.

The apparent link in our experiments between the growth factor independence and gross karyotypic changes may result from the recessive nature of the tumorigenic phenotype (2, 5, 6, 12). Unbalanced genomes caused by an abnormal distribution of chromosomes to the daughter cells after crisis may allow the expression of a recessive trait.

Lee et al. (7) have shown that the transformation of normal rat embryo cells by cotransfection with *c-myc* and EJ *Ha-ras* DNAs was contingent on overexpression of *c-myc*. All our cultures infected with *v-myc* virus showed a high incidence of cell death, presumably caused by a toxic effect of the overproduced *p57^{v-myc}* protein. The question arises whether this toxic effect of the overproduced *p57^{v-myc}* protein plays a role in the observed karyotypic changes. Our results (Fig. 3d) show a striking similarity with results in experiments described by Zajac-Kaye and Ts'o (15), in which Syrian hamster embryo cells were exposed to DNAase 1 encapsulated in liposomes; the treatment was toxic. Gross karyotypic changes were observed after crisis. A cell line obtained after crisis showed a major fraction (64%) of cells with near-tetraploid and -octoploid karyotypes. All eight DNAase-treated cultures became established cell lines in contrast with only 6.3% of the untreated cultures. In the present experiment, 85% of the cells of a cloned population became near-tetraploid and octoploid at the time of crisis; all 11 cloned populations studied came out of crisis and gave rise to growth factor-independent cell lines.

This study shows that cells coinfecting with viruses containing the *v-ras* and *v-myc* oncogenes became growth factor

independent only after a lag of 2 to 3 months after the cells had gone through crisis. All growth factor-independent cells showed a great variability in their karyotypes. It is conceivable that the expression of growth factor independence is due to specific chromosomal changes, the nature of which is central to our understanding of the process which leads to a fully tumorigenic cell.

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