raf/*myc*-Infected Erythroid Cells Are Restricted in Their Ability To Terminally Differentiate

S. PETER KLINKEN,^{1,2*} ULF R. RAPP,³ AND HERBERT C. MORSE III¹

Laboratory of Immunopathology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892¹; The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Parkville, Victoria, 3050, Australia²; and Laboratory of Viral Carcinogenesis, Frederick Cancer Research Facility, National Cancer Institute, Frederick, Maryland 21701³

Received 23 June 1988/Accepted 8 November 1988

A comparison was made of the in vitro erythroid colony-forming abilities of *v-raf*-, *v-myc*-, and *v-raf/v-myc*-containing retroviruses. In methylcellulose, *v-raf* efficiently produced colonies of well-differentiated hemoglobin-synthesizing erythroid cells, whereas *v-raf/v-myc*-infected erythroid cells were inhibited from terminally differentiating but retained the ability to replicate extensively. In contrast, *v-myc* was unable to stimulate the formation of erythroid colonies.

3611-MSV, containing the *v-raf* oncogene, produces hemopoietic neoplasms confined almost exclusively to the erythroid lineage, while a retroviral construct (J2) expressing *v-raf* and avian *v-myc* induces lymphoblastic lymphomas, as well as erythroblastosis (17, 18). Retroviruses J3 and J5, containing functional *v-myc* genes (17), generate B and T lymphomas in vivo (16). In this study, we compared the effects of 3611-MSV, J2, J3, and J5 on erythroid cells in culture to determine whether the virus-induced erythroproliferation observed in mice could be reproduced in vitro. This would facilitate investigations on target cells for the individual viruses and on hormone responsiveness of the infected cells, which could lead to treatment of the tumors in vivo.

Bone marrow from phenylhydrazine-treated mice provided a source of hemopoietic cells enriched for erythroid progenitors. The cells were infected with equivalent amounts of each virus and plated in 0.75% methylcellulose containing 0.1 U of erythropoietin (Epo) (Connaught Medical Laboratories, Toronto, Canada) per ml by the method of Hankins et al. (11). Under these conditions, only virus-infected colonies develop (11-14), since 10- to 20-fold-higher concentrations of Epo are required to stimulate normal erythroid precursors into colony formation (8). Colonies of erythroid cells emerged from 3611-MSV- and J2-infected bone marrow cultures but at very low frequencies (Table 1). Nevertheless, the appearance of erythroid colonies following infection with 3611-MSV and J2, but not J3 and J5, accurately reflects the erythroblastosis observed in vivo (16-18) and demonstrates the efficacy of the in vitro assay system.

To enhance erythroid colony formation, we used fetal liver cells to provide target cells for the retroviruses (20). The data in Table 1 indicate that there were far more erythroid target cells in fetal liver than in bone marrow. The numbers of erythroid colonies produced by 3611-MSV and J2 infection were fairly similar, with no more than 45% difference separating the two groups in six separate experiments. This lack of cooperativity between *v-raf* and *v-myc* was in marked contrast with transformation of lymphocytes in agarose, in which the two oncogenes acted in synergy to

increase lymphoid transformants 14-fold (S. P. Klinken et al., submitted for publication).

The 3611-MSV-induced erythroid colonies were small, compact, and highly hemoglobinized (Fig. 1A). By comparison, the vast majority of J2-stimulated colonies were very large, containing approximately five times as many cells as the 3611-MSV colonies (Fig. 1B). In addition, the large J2 colonies were poorly hemoglobinized, with less than 5% of the cells reacting with the benzidine stain. Cytocentrifuge preparations of these colonies indicated that the 3611-MSV-infected cells (Fig. 1D) contained large numbers of hemoglobin-synthesizing polychromatophilic and orthochromatophilic erythroblasts. In contrast, more than 95% of the cells from J2 colonies were proerythroblasts and basophilic erythroblasts, the early non-hemoglobin-producing cells of the erythroid series (Fig. 1E). It is noteworthy that the J2-induced erythroblastosis in vivo is represented mainly by basophilic erythroblasts (17).

J3 infection of fetal liver cells did not result in erythroid colony formation; myeloid colonies developed instead, albeit at a very low frequency (Table 1). The colonies were fairly large, irregular (Fig. 1C), and composed mainly of myelocytes and metamyelocytes, the immature cells of the myeloid lineage (Fig. 1F). These results are comparable with the data of Brightman et al. (5) and Baumbach et al. (2), who observed myeloid colony formation in vitro with *v-myc*- and *c-myc*-containing viruses, respectively. The induction of lymphoid colonies with J3, commensurate with the in vivo disease (16), was observed only in agarose (Klinken et al., submitted).

To determine whether the virus-infected erythroid cells responded to concentrations of Epo higher than 0.1 U/ml, we incubated fetal liver cells with the virus and then plated them in methylcellulose with increasing amounts of hormone. Raising the concentration of Epo did stimulate hemoglobin synthesis in the J2-induced colonies (Fig. 2A). Nevertheless, distinct differences in Epo responsiveness were apparent between the colonies produced by the *v-raf* and *v-raf/v-myc* viruses. (i) 3611-MSV-infected cells responded to 0.1 U of Epo per ml, whereas 0.5 U of Epo per ml was necessary to stimulate hemoglobinization of J2-infected cells. (ii) More than 90% of *v-raf*-induced colonies became benzidine positive with Epo, but only 50% of the *v-raf/v-myc* colonies responded to the hormone. (iii) Almost all the cells in

* Corresponding author.

TABLE 1. Erythroid colony formation with 3611-MSV, J2, J3, and J5 viruses^a

Virus	Colony no./ 10 ⁶ cells		Cell no./ colony	Benzidine reactivity ^b
	BM	FL		
Control (uninfected)	0	0		—
3611-MSV	6	812	~2,000	+++
J2	5	697	~10,000	±
J3	ND ^c	20	2–4,000	—
J5	0	0		—

^a Bone marrow cells (BM) (4×10^6) from phenylhydrazine-treated NFS/N mice or 10^6 fetal liver (FL) cells from 12-day-old NFS/N fetuses were incubated with the viruses and then plated in methylcellulose with 0.1 U of Epo per ml. Colonies were scored 7 days later and stained with benzidine-hematoxylin.

^b Number of cells reacting with benzidine stain: —, <1%; ±, <5%; +, +, +, >90%.

^c ND, Not determined.

3611-MSV colonies made hemoglobin compared with 40% or less in J2 colonies. Therefore, unlike 3611-MSV-infected erythroid cells, only a proportion (~20%) of J2-infected cells responded to Epo and these cells appeared less sensitive to the hormone. This result suggests that the *raf*-induced erythroblastosis *in vivo* may be alleviated by Epo treatment, enabling infected cells to terminally differentiate. However, it appears unlikely that this approach would work with erythroid cells transformed by J2. Interestingly, superinfection with *v-abl* did not induce hemoglobin synthesis in either *v-raf* or *v-raf/v-myc* colonies (data not shown) as it does with *v-Ha-ras*-infected cells (19).

As the maximum number of colonies for both 3611-MSV and J2 occurred between 6 and 8 days postinfection (Fig. 2B), it was postulated that these viruses may be affecting similar progenitor cells, the 6- to 8-day BFU-E cells (12–14). To test this hypothesis, we infected fetal liver cells with 3611-MSV or J2 alone or with both viruses together. No significant differences in colony numbers were detected between the three groups (Table 2), suggesting that in fetal liver there exists a limited pool of target cells which the two viruses share and that the obvious differences in phenotype between *v-raf*- and *v-raf/v-myc*-induced colonies must be due to the introduced *v-myc* gene. This result emphasizes the fact that the introduced genes direct the phenotype of the cell and that the differences do not reside with different target cells. Similarly, Waneck et al. (19) demonstrated that the phenotype of *v-Ha-ras*-infected erythroid cells can be altered by the addition of *v-abl*, thus allowing the cells to differentiate in the absence of Epo.

The data presented here indicate that infection of hemopoietic precursors with 3611-MSV or J2 in methylcellulose produces colonies of erythroid cells which differ markedly in their ability to replicate, differentiate, and respond to Epo. The 3611-MSV-infected erythroid cells reacted readily to low concentrations of exogenous Epo and differentiated into hemoglobin-synthesizing cells; these effects of *v-raf* are very similar to those induced by Friend virus (anemia-inducing strain) and retroviruses expressing *ras*, *mos*, *fes*, *erbB*, *cbl*, and *src* oncogenes (1, 11–14). In contrast, the *v-raf/v-myc*-containing erythroid cells had an enhanced ability to divide but were restricted to their ability to terminally differentiate. Furthermore, continuous erythroid cell lines

FIG. 1. Colony formation with 3611-MSV, J2, and J3 viruses. Fetal liver cells were incubated with the viruses and then plated in methylcellulose with 0.1 U of Epo per ml. (A) 3611-MSV-induced erythroid colony; (B) J2-induced erythroid colony; (C) two J3-induced myeloid colonies ($\times 125$). Cytocentrifuge preparations from 3611-MSV (D), J2 (E), and J3 (F) colonies above were stained with Wright-Giemsa stain ($\times 1,000$). Abbreviations: p, proerythroblast; b, basophilic erythroblast; pc, polychromatophilic erythroblast; o, orthochromatophilic erythroblast; m, myelocyte; mm, metamyelocyte.

FIG. 2. (A) Response of 3611-MSV and J2 cells to Epo. Fetal liver cells (10^6) were incubated with the viruses and then cultured in methylcellulose with various amounts of Epo. The number of benzidine-positive colonies was determined 7 days later and expressed as a percentage of the total colony numbers (figures in parentheses above each point). (B) Time course of colony formation with 3611-MSV and J2. Fetal liver cells were exposed to 3611-MSV and J2 and plated in methylcellulose with 0.1 U of Epo per ml, and total colony numbers were determined daily.

have recently been generated with the J2 construct, and only a proportion of these cells differentiate with Epo (14a).

A striking analogy can be drawn between the effect of *v-raf/v-myc* on primary erythroid cells and the inability of Friend murine erythroleukemia cell lines transfected with *c-myc* to terminally differentiate (6, 7, 15). The erythroid cells from both groups contain novel, dysregulated *myc* genes and are restricted in their capacity to terminally differentiate as the majority of cells continue to replicate in the presence of their inducers and only a small percentage of

cells become benzidine positive. It would appear that constitutive expression of *myc* sequences in erythroid cells alters the cellular sensitivity to differentiation signals, supports proliferation, and inhibits terminal differentiation. However, in contrast with the failure of long-term erythroleukemia cell lines transfected with *c-myc* to respond to xenobiotic inducers of differentiation, we demonstrated here that the addition of *v-myc* to primary *v-raf* erythroid transformants results in reduced responsiveness to Epo, their normal physiological regulator.

An interesting comparison can also be made between J2 and avian erythroblastosis virus and the effects of these viruses on erythroid cells. Both viruses contain oncogenes for a kinase and a nuclear protein; J2 has a serine-threonine kinase (*v-raf*) and *v-myc*, while avian erythroblastosis virus contains *v-erbA* and *v-erbB*. Both viruses infect early erythroid progenitors, providing extensive self-renewal capacity but restricting terminal differentiation (3, 4, 9, 10). The apparent functional equivalence between *v-myc* and *v-erbA* on the one hand and *v-raf* and *v-erbB* on the other presumably reflects overlaps in oncogene signaling pathways such that a common set of target genes, for example, band 3 (21), is altered in expression.

We thank Sandra Ruscetti and Greg Johnson for assistance with the erythroid colony-forming assay.

This work was supported in part by a grant from the National Health and Medical Research Council (Australia) and by Public Health Service grant CA 12421 from the National Cancer Institute.

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TABLE 2. Effect of using viruses in combination^a

Virus(es)	Benzidine-positive colonies/10 ⁶ cells	Total colonies/10 ⁶ cells
Control (uninfected)	0	0
3611-MSV	552	620
J2	46	761
3611-MSV + J2	273	682

^a Fetal liver cells were incubated with equal titers of 3611-MSV or J2 individually or with both viruses simultaneously. Colonies were scored after growth for 1 week in methylcellulose with 0.1 U of Epo per ml.

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