

Role of Cellular RNA Polymerases in Virus-induced Leukemogenesis¹

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SUMMARY

Friend murine leukemia virus induces splenic enlargement and an increase in RNA polymerase activity of spleen nuclei. Actinomycin D, administered at 60 $\mu\text{g}/\text{kg}$ body weight/day prevents the development of splenomegaly and the elevation of polymerase activity following infection, but it has only a slight effect on the production of virus in spleen tissue. Thus, the alteration of RNA synthesis is not a result of virus proliferation, but instead may be a manifestation of leukemic erythropoiesis. Normal erythropoiesis, stimulated by erythropoietin administration, produces a similar but transient increase in RNA polymerase activity in spleen nuclei. Erythropoietin administered before, but not after, Friend virus infection results in an enhancement of RNA polymerase activity, as measured 9 days after inoculation. This effect is most simply explained by assuming that there is a common target cell pool for both erythropoietin and Friend virus, and that this pool becomes refractory to the influence of the hormone as a result of the leukemic process.

INTRODUCTION

During mammalian development, the major site of red blood cell production shifts from the embryonic yolk sac, to the fetal liver, and finally to the bone marrow (13). In the adult mouse, the spleen retains some hemopoietic capacity (2, 6), which is controlled by the glycoprotein hormone, erythropoietin. Erythropoiesis in the mouse spleen can be stimulated by direct administration of erythropoietin (14) or by hypoxia- or anemia-induced elevation of endogenous levels of the hormone (19).

Stimulation of erythropoiesis in the spleen activates key enzymes in heme biosynthesis and results in increased ⁵⁹Fe uptake by hematopoietic tissue. Similar responses are associated with the virus-induced, erythroblastic proliferation of murine leukemia which follows infection with the Rauscher or F-MuLV³ (7, 8).

To elucidate the role of erythropoiesis in the leukemic

process, we investigated one of the earliest responses to erythropoietic stimulation—an increase in rRNA polymerase activity (5, 9, 34). Since F-MuLV infection stimulates RNA synthesis in erythropoietic tissue (1, 15, 21, 33), it was of particular interest to determine whether this change is associated with the cellular proliferation caused by F-MuLV infection, or with the virus replication *per se* occurring in infected spleen cells.

MATERIALS AND METHODS

Friend Virus Infection in Mice. Random-bred (21-day-old) ICR/Ha Swiss mice from the colony maintained in this laboratory, were inoculated i.p. with a leukemogenic dose of Friend virus, using protocols previously established (27). At various times after inoculation, the mice were killed by cervical dislocation and the spleens were quickly excised. The organs were weighed and placed on ice for not longer than 1 hr before use. Appropriate dilutions of a 20% extract of spleens (w/v) were assayed for infectious virus activity in recipient mice as previously described (27). Virus titers are expressed as LD₅₀, as determined by the method of Reed and Muench (24). Mice from the same colony served as donors of red blood cells for hypertransfusion studies.

Nuclei Preparation and RNA Polymerase Assay. Nuclei were isolated from spleen homogenates by sucrose density gradient centrifugation according to established techniques (1). The concentration of nuclei in the final suspension was determined with a Coulter cell counter. The standard polymerase assay contained: 50 mM Tris-Cl (pH 7.9); 2 mM MnCl₂; 0.5 mM each ATP, GTP, and CTP; 0.05 mM [2-¹⁴C]UTP, 100 cpm/pmole; 80 mM (NH₄)₂SO₄; 1 mM dithiothreitol; 5 mM phosphoenolpyruvate; 1 μg pyruvate kinase; and 1 to 5 $\times 10^7$ nuclei, in a total volume of 0.2 ml. One unit of activity is the incorporation of 1 pmole of [¹⁴C]UTP into an acid-insoluble product during 10-min of incubation at 37°, determined as previously described (1).

Materials. Reagents for the RNA polymerase assay were from sources detailed previously (1). Actinomycin D was acquired from Merck, Sharp and Dohme, Rahway, N. J., and erythropoietin (step III preparation) was from Connaught Laboratories, Toronto, Canada.

Statistical Treatment of Experimental Data. The spleen weights and RNA polymerase activity from the 7-day experiment shown in Table 2 were examined by a factorial analysis of variance. The data from the 10-day experiment of Table 2 and that found in Table 3 were analyzed by a

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³ The abbreviations used are: F-MuLV, Friend murine leukemia virus; LD₅₀, leukemia dose, 50%.

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Duncan multiple-range test. The mean of the spleen weights of those animals constituting a pool for nuclei preparation was analyzed as a single variant.

RESULTS

RNA Polymerase and Virus Replication. The random-bred ICR/Ha Swiss mice used in this study respond to F-MuLV infection by a sharp increase in the RNA polymerase activity of spleen tissue (1, 15). Spleen weight and the RNA polymerase of nuclei prepared from spleen tissue have nearly doubled by 7 to 10 days after i.p. injection of 320 LD₅₀ of F-MuLV. The spleen weight continues to increase until at least Day 14 postinfection (32), and the polymerase activity remains elevated (Fig. 1; Ref. 1).

Table 1 shows the characteristic of RNA synthesis by spleen nuclei isolated from normal and F-MuLV-infected mice. The incorporation was dependent on the presence of all 4 nucleotide triphosphates, and nuclei from normal and infected tissue were comparably inhibited by the inclusion of actinomycin D into the assay, suggesting that the product was a heteropolymer transcript. Synthesis under the conditions of this assay was approximately 70% resistant to α -amanitin, indicating the predominance of nucleolar RNA polymerase I activity (1).

It has been shown that actinomycin D treatment of mice infected with erythrocytic leukemia virus reverses the

splenomegaly induced by these agents. To determine the therapeutic effect of the drug on RNA polymerase activity, virus proliferation and spleen weight, the experiment summarized in Table 2 was performed.

Spleen weights in actinomycin D-treated, virus-inoculated mice were lower than those of untreated, virus-infected mice at both 7 and 10 days after inoculation. Similarly, at both 7 and 10 days, the elevated RNA polymerase activity that normally followed F-MuLV infection was effectively prevented by actinomycin D treatment. A factorial analysis of variance showed that the effects of actinomycin D and F-MuLV on spleen weight and polymerase activity, as well as their interaction were significant at $p < 0.05$.

In contrast to the inhibitory effects of actinomycin on splenomegaly, the drug had no discernible effect (after 7 days) on the proliferation of F-MuLV in the spleen. Even at 10 days, only a small decrease was observed in the virus titer of spleen tissue from actinomycin D-treated mice. A similar dissociation of virus replication and leukemogenic cellular alterations has been observed in genetically resistant mice (17, 23). Furthermore, under *in vitro* culture conditions, where infected mouse embryo fibroblasts actively synthesize F-MuLV but demonstrate no perceptible pathophysiological responses characteristic of leukemogenesis, no elevation in RNA polymerase activity could be detected (29) (and unpublished observations of D. F. Babcock). Hence, since the F-MuLV-induced increase in RNA polymerase activity does not seem to be a requirement for virus replication, it must instead be part of the leukemogenic response of the host tissue.

RNA Polymerase and Erythropoiesis. To clarify the relationship between erythropoiesis and RNA polymerase activity in the leukemogenic process, we compared the effects of hypertransfusion, erythropoietin administration, and F-MuLV infection on the RNA polymerase activity of murine spleen cells (Table 3). Endogenous erythropoiesis was suppressed by hypertransfusion in 5 sets of 10 to 15 mice (Groups A to E). At Day 7, Groups B, D, E, and F were infected with F-MuLV. In Group C, erythropoiesis was restimulated by injections of erythropoietin at 24 and 12 hr before sacrifice on Day 16. Erythropoietin treatment

Table 1
Characteristics of RNA synthesis by isolated spleen nuclei

Assay	Polymerase activity (units/10 ⁷ nuclei) with nuclei source	
	Normal	Infected ^a
Complete	6.4	10.4
Minus ATP	1.2	1.7
Plus α -amanitin (1 μ g)	4.2	6.7
Plus actinomycin D (10 μ g/mg DNA)	1.2	1.9
Plus actinomycin D (100 μ g/mg DNA)	0.3	0.5

^a Ten days after i.p. injection of 300 LD₅₀ of F-MuLV.

Table 2
Effect of actinomycin D on F-MuLV-induced RNA polymerase and F-MuLV replication

Treatment ^a	Spleen wt ^b (g)		Polymerase activity ^{b, c} (units/10 ⁷ nuclei)		Virus synthesis ^d (titer)	
	7 days	10 days	7 days	10 days	7 days	10 days
None	0.19 ± 0.007	0.21 ± 0.006	6.8 ± 0.3	6.8 ± 0.3		
Actinomycin D ^e	0.11 ± 0.005	Not Done	6.7 ± 0.2	Not Done		
F-MuLV ^f	0.31 ± 0.03	0.66 ± 0.06 ^g	9.5 ± 0.2	13.3 ± 0.1 ^h	10 ^{2.67}	10 ^{2.88}
F-MuLV ^f + Actinomycin D ^e	0.15 ± 0.01	0.15 ± 0.01	7.0 ± 0.2	7.4 ± 0.1	10 ^{2.67}	10 ^{2.88}
Error mean square	0.00060	0.0011	0.159	0.123		

^a Each group contained 27 mice.

^b Mean ± S.E.

^c Nuclei from 3 separate pools (of 3 to 5 spleens each) were assayed for polymerase activity.

^d Virus content of pooled spleen extract from 6 mice (by infectivity assay) is expressed as LD₅₀/100-mg spleen.

^e Actinomycin D (60 μ g/kg body weight) was administered s.c. in 0.2 ml at 24-hr intervals, beginning 12 hr postinoculation.

^f Inoculation with 320 LD₅₀ of F-MuLV.

^g Values determined for this group differed from those of all other groups at the 1% confidence limit.

Table 3
Effect of hypertransfusion and erythropoietin on F-MuLV-induced leukemia

Group ^a	Treatment on Day:																Polymerase activity ^{b, c} (units/10 ⁷ nuclei)	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
A											HP ^d	HP				Harvest	0.21 ± 0.03	6.5 ± 0.2
B		HP	HP				F-MuLV ^{e, f}									Harvest	0.36 ± 0.03	8.2 ± 0.2
C											HP	HP			EP ^g	Harvest	0.26 ± 0.01	8.6 ± 0.1
D		HP	HP			EP ^g	F-MuLV									Harvest	0.41 ± 0.07	12.1 ± 0.3
E			HP				F-MuLV ^r				EP					Harvest	0.38 ± 0.09	7.5 ± 0.2
F							F-MuLV									Harvest	0.40 ± 0.05	8.5 ± 0.2
G																Harvest ^r	0.18 ± 0.01	6.0 ± 0.01
Error mean square																	0.0081	0.1009

^a Each group contained 10 to 15 mice.
^b Mean ± S.E. At the 5% confidence limit, spleen weights of Group A + Groups B, D, E, F; of Group C + Groups A, G; of Group C + Groups D, E, F, G; of Groups D, E, F + Groups A, C, G and the polymerase activity of Groups A, G + Groups B, C, D, E, F; of Groups B, C, F + Groups A, D, E, G; of Group D + Groups A, B, C, E, F, G; of Group E + Groups A, B, C, D, F, G.
^c See Table 2, Footnote c.
^d Hypertransfusion (by i.p. injection) of 1 ml packed red blood cells.
^e Animals with hematocrits less than 65% were not further utilized.
^f See Table 2, Footnote g.
^g Erythropoietin injections of 1 unit each (s.c.) were administered 12 and 24 hr before subsequent treatment.

preceded (Group D) or followed (Group E) F-MuLV infection. Two nonhypertransfused control groups were established—an infected group (F) and an uninfected group (G). All animals were killed on Day 16, counting from the day of the earliest hypertransfusion (Group D).

By 9 days after infection, the RNA polymerase activity of the infected controls (Group F) was elevated by 40%, and the average spleen weight was twice that of the uninfected controls (Group G). Hypertransfusion alone had no effect on either spleen weight or polymerase activity, as measured 4 days after treatment in the uninfected (Group A) or hypertransfused, infected (Group B) animals. Table 3 shows that administration of erythropoietin to hypertransfused animals shortly before sacrifice (Group C) causes significant increases in splenomegaly and in polymerase activity. The transient influence of erythropoietin on erythropoiesis in the mouse is well established (11, 22) and was given major consideration in the design of this experiment. Coadministration of erythropoietin was scheduled so that only the potentiating effects of the hormone on F-MuLV-induced leukemogenesis would be observed.

The data in Table 3 further show that the effect of erythropoietin on F-MuLV-induced splenomegaly is marginal. However, its enhancement of the accompanying elevation of splenic RNA polymerase activity that accompanies F-MuLV infection is significant. RNA polymerase activity of nuclei prepared from such tissue (Group D) 10 days after administration of erythropoietin was nearly 50% higher than that of a comparable preparation from infected animals (Group B) that did not receive the hormone. In contrast, when erythropoietin was administered 4 days after F-MuLV infection (Group E), this effect on leukemogenesis was not observed. Spleen size and RNA polymerase activity of this group were not different than those of the control (Group B).

DISCUSSION

Studies of erythroid precursor populations following stimulation of normal erythropoiesis have shown that erythropoietin causes a particular population of cells, responsive to this hormone, to differentiate and, more important, to proliferate (3, 5, 10, 11, 26-28). This process is reversibly blocked by low levels of actinomycin D (25). That the target cells for both F-MuLV and erythropoietin may be the same is suggested by the enhancing effect of erythropoietin on leukemogenesis, as measured by histological techniques (18, 35). The suppression of F-MuLV-induced splenomegaly by comparably low dosages of actinomycin D seen here (Table 2) and by others (4, 36) supports this contention. The rapid decay of erythropoietin-induced RNA synthesis in the normal mouse spleen documented by others (11, 22) precludes a simple additive explanation for the enhancement by erythropoietin of the F-MuLV-stimulated RNA polymerase activity observed in this study. The potentiating effect of prior erythropoietin administration on virus-induced changes in RNA synthesis shown in Table 3 is most simply explained by assuming that

there is an enlargement of a target-cell pool responsive both to erythropoietin and to virus infection. Other interpretations are of course possible.

This study, while not conclusive, does suggest that the increase in RNA polymerase activity that follows F-MuLV infection is similar to that induced by erythropoietin. Actinomycin treatment prevents the virus-promoted increase in RNA synthetic ability (Table 2) as it does for erythropoietin-induced responses (25, 34). The additivity of the effects of erythropoietin and F-MuLV infection resembles cooperativity observed with multiple injections of erythropoietin (11). The magnitudes of the changes induced by the hormone and by virus infection are comparable (Table 3). Moreover, the molecular mechanisms for enhanced RNA synthesis have been shown to involve a preferential increase in the activity of a nucleolar RNA polymerase in response to F-MuLV (1) or erythropoietin (34). This activation of RNA synthesis precedes other changes in macromolecular synthesis in erythropoietin-stimulated tissues (5, 9) and results in increased production of (guanosine-cytosine rich) pre-rRNA in spleen nuclei following erythropoietin administration (34) or F-MuLV infection (15).

Oncornavirus RNA-dependent DNA polymerase is necessary for the initiation, but not the maintenance, of virus replication (12, 16, 37), which is instead probably mediated by the host cell nucleoplasmic RNA polymerase (1, 28). The lesser sensitivity of these 2 enzyme activities to actinomycin D (1, 10), compared with nucleolar RNA polymerase, may explain the resistance of virus replication in spleen tissue of mice treated with the low antibiotic levels (*cf.* Refs. 4, 31) administered in these studies. The extreme susceptibility of normal and leukemic erythropoiesis to actinomycin D may relate to the well-known preferential inhibition of preribosomal RNA synthesis by low levels of this drug.

We propose then that F-MuLV infection and erythropoietin may cause a similar initial alteration of RNA synthesis. However, the later divergence of normal and leukemic erythropoiesis is indicated by the fact that, following the initiation of the leukemogenic process, erythropoietin administration no longer enhances the virus-induced elevation of RNA polymerase activity (See Table 3, Groups D and E). The failure of antierythropoietin antiserum to suppress F-MuLV-induced erythropoiesis (20) and the absence of erythropoietin activity in the plasma of F-MuLV infected mice (20, 30) also indicate that leukemogenic erythropoiesis may be manifested by loss of control of nucleolar RNA synthesis, an event closely related to the primary action of erythropoietin. It is hoped that an extension of the studies reported here may provide further understanding of this loss of hormonal control as a critical step in the leukemogenic process.

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