Potentiation of the Erythropoietin Response by Dimethyl Sulfoxide Priming of Erythroleukemia Cells: Evidence for Interaction of Two Signaling Pathways

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Erythropoietin (Epo) and dimethyl sulfoxide (DMSO) are believed to induce the differentiation of transformed erythroid cells by different signal transduction pathways. We have now obtained evidence for the interaction of these pathways. We used a Rauscher murine erythroleukemia cell line with a relatively low (8% to 10%) hemoglobinization response to Epo alone. Pretreatment of these cells for 1 day with DMSO followed by its removal and the addition of Epo resulted in a marked enhancement of the Epo specific hemoglobinization. We have designated this effect "DMSO priming." This priming effect of DMSO on the Epo response was both time-dependent and DMSO concentration-dependent. DMSO priming potentiated the Epo response in three ways. Firstly, DMSO priming increased the total number of Epo responsive cells from 8% to 10% to 40% to 60%. Secondly, DMSO priming reduced the time required to reach the optimal Epo-induced response from 4 days to 2 days. Thirdly, the Epo dose-response curve was left-shifted approximately 20-fold. DMSO priming was also associated with a marked increase in Epo receptor density characterized by an apparently new receptor population and by the appearance of positive cooperativity between receptors. Our results suggest that the DMSO priming effect is due to potentiation of the Epo signaling pathway, thus resulting in a much more rapid and dramatic Epo-induced hemoglobinization response.

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A COMPLETE understanding of the regulation of erythropoiesis requires the identification of the humoral and cellular factors involved in this regulation and their modes of action. No single experimental model has yet been derived that permits the study of all such regulatory influences.

In 1971, Friend et al. demonstrated that dimethyl sulfoxide (DMSO) caused terminal differentiation in vitro of virus-induced murine erythroleukemia cells. This "Friend cell" model system has allowed the dissection of several aspects of erythropoiesis on a molecular level. The salient disadvantage of these cells has been their lack of response to erythropoietin (Epo).* However, in 1979 DeBoth et al. derived a continuous cell line from the leukemia cells of Rauscher virus infected mice. In contrast to Friend cells, these "Rauscher cells" differentiated in response to both Epo and DMSO. In analyzing clonal lines derived from the original Rauscher murine erythroleukemia cell line, we found that the responses to Epo and DMSO could be segregated. We proposed that Epo and DMSO induced erythroid differentiation by different mechanisms. We have found that Epo and DMSO have other differential effects on Rauscher cells. For example, Epo, but not DMSO, induces new β-adrenergic receptors, up-regulates an erythroid-specific membrane determinant, ERY-1, and causes a rapid dephosphorylation of pp43, an erythroid membrane protein.

In studying the signal transduction pathway triggered by the Epo-receptor interaction, we were prompted to examine the simultaneous effect of Epo and DMSO on the differentiation of clonal Rauscher lines. The results of these studies indicate that DMSO strongly amplifies the Epo response of these cells, suggesting that the signaling pathways of these two inducers interact.

MATERIALS AND METHODS

Cell culture and induction studies. R28 was subcloned from a primary Rauscher murine erythroleukemia cell line by limiting dilution. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY). To induce differentiation, DMSO (Sigma, St Louis, MO) or highly purified recombinant human Epo (Elanex Pharmaceuticals, Inc, Bothell, WA) was added to the medium. In some experiments, as noted in the Results section, DMSO was "removed" by repeated washing and centrifugation of cells in DMEM-free medium followed by replating in the absence of DMSO. The effect of DMSO treatment on cell proliferation (total number of cells) was examined by performing daily cell counts on replicate cultures grown for specified times in the absence or presence of DMSO (see Results). Hemoglobinized cells (Hb+) were assayed using benzidine staining. Assays were performed by adding 10 μL freshly prepared benzidine reagent containing 0.6% H2O2, 0.5 mol/L CH3COOH, and 0.2% benzidine dihydrochloride to 50 μL of cells (0.5 to 1 x 10⁶ cells/mL) in culture medium. The proportion of benzidine positive cells (blue cells) was scored out of 200 cells counted and is expressed as "Hb+ cells, %". Variation between duplicate counts and repeat experiments was usually less than 5%.

Radioiodinated Epo. Recombinant human Epo (Elanex) was labeled using IODGEN (Pierce) and carrier-free ¹²⁵I (American; 174 mCi/μg iodine; 644 MBq/μg). ¹²⁵I-labeled Epo was purified using BioGel P6DG (BioRad) gel filtration in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (Sigma) and 0.02% Tween-20 (Sigma). The specific radioactivity of ¹²⁵I-labeled Epo ranged from 0.1 to 0.4 gram atom/mmol for different preparations. Iodinated Epo were prepared using this method retained full biologic activity when assayed by an in vitro bioassay.

Binding of iodinated Epo to cells. Cells were harvested by centrifugation, washed twice in Dulbecco's PBS, and incubated in DMEM containing 10% FBS and 0.2% sodium azide (binding
medium) for 30 minutes. Triplicate samples of \(5 \times 10^6\) cells in 200 
\(\mu L\) of binding medium containing \(^{125}\)I-Epo at specified concentrations were incubated in the absence or presence of 100-fold unlabeled Epo at 37°C for 30 minutes. At the end of the incubation, cells were transferred to microfuge tubes containing 200 
\(\mu L\) of FBS, centrifuged through the cushion of FBS for 5 minutes in a Beckman MICROFUGE 12, and frozen at \(-80°C\). The tip of the tube containing the cell pellet was cut off. Radioactivity in the cell pellet was calculated from specific binding (cpm) by using the following constants: carrier-free \(^{125}\)I = \(2.2 \times 10^{10}\) Ci/gram atom; Ci = \(2.2 \times 10^{12}\) dpm; 83% efficiency of Gamma 5500 counter determined experimentally (1 cpm = 1.2 dpm); specific activity of \(^{125}\)I-Epo used in binding study shown equals 0.12 gram atom iodine/mol Epo.

RESULTS

Synergistic effect of DMSO and Epo on hemoglobinization of Rauscher cells. We examined the effect of Epo and DMSO on the hemoglobinization of several Rauscher cell clones in suspension culture. As we have observed in plasma clot culture, different clones exhibited characteristic responses to the two inducers in terms of \(Hb^{+}\) cells as percent of total. One subclone, R28, which exhibited a low to moderate response to either DMSO or Epo, was selected for further examination. DMSO and Epo induced hemoglobinization of R28 to 33% and 8%, respectively, after 3 days of exposure to the inducers (Fig 1). However, when the cells were exposed to both inducers simultaneously, a striking synergistic effect was observed. After only 2 days, 70% of the cells were hemoglobinized, while less than 10% of the cells were hemoglobinized when each inducer was used alone for this time period. A similar synergistic effect between DMSO and Epo was observed in two other clones of Rauscher cells examined (data not shown).

We considered the possibility that DMSO treatment might be inhibiting the growth of some cells, thereby allowing a preferentially Epo-responsive subpopulation to proliferate. We had shown previously that DMSO exhibited a moderate growth inhibitory effect on some Rauscher cell clones when the agent was present in the culture for 3 days.

Therefore, we tested this possibility by establishing three replicate cultures of 150,000 cells/mL and incubating them as follows: culture A, no DMSO; culture B, 24 hours with 1% DMSO followed by 24 hours without DMSO; culture C, 48 hours with 1% DMSO. After incubation, the total cell counts were: culture A = \(3.1 \times 10^6 \pm 0.5 \times 10^6\) cells/mL; culture B = \(3.0 \times 10^6 \pm 0.5 \times 10^6\) cells/mL; culture C = \(2.3 \times 10^6 \pm 0.3 \times 10^6\) cells/mL. These results indicate that the negligible effect of DMSO on growth is insufficient to explain the large increase in Epo response.

 Pretreatment with DMSO (DMSO priming) increases the Epo response of R28 cells. We next investigated whether the simultaneous presence of both inducers was necessary for this synergistic effect. Rauscher cells were pretreated with 1% DMSO for 24 hours, washed twice, and then incubated in the absence (Epo-) or presence (Epo+) of Epo (10 U/mL) (Fig 2). One day after the DMSO was removed, the Epo- cells were 28% \(Hb^{+}\), whereas the Epo+ cells were 56% \(Hb^{+}\) (28% Epo-specific \(Hb^{+}\) cells). Two days after DMSO removal, the Epo- cells were only 6% \(Hb^{+}\) while the Epo+ cells were 45% \(Hb^{+}\) (35% Epo-specific \(Hb^{+}\)). This Epo response compares with only 1% \(Hb^{+}\) after 48 hours of Epo treatment without DMSO pretreatment (Fig 1). Thus, pretreatment with DMSO followed by its removal was nearly as efficacious as amplifying the Epo response as was simultaneous addition of the two inducers. We have designated pretreatment of cells with DMSO followed by its removal before addition of Epo as "DMSO priming."
**DMSO priming is time-dependent and concentration-dependent.** To characterize "DMSO priming" further, cells were pretreated with 1% DMSO for various periods of time. DMSO was removed, and Epo was added to the medium for 24 hours. The percentage of hemoglobinized cells was then assessed (Fig 3A). A slight amplification of the Epo response was seen after only 6 hours of priming, and a clear effect was observed after 12 hours. Maximal amplification occurred after 48 hours of priming. The dose-response of "DMSO priming" was examined next by pretreating R28 cells with different concentrations of DMSO for 24 hours. After removing DMSO, Epo was added to the cells for 24 hours. We observed an almost linear relationship between the Epo specific hemoglobinization and the concentration of DMSO used for priming (Fig 3B).

**DMSO priming increases the number of Epo-responsive cells and the rate of Epo response.** The striking effect of DMSO priming on the number of Epo responsive cells is presented in Fig 4A. In this experiment, R28 cells were pretreated with 1% DMSO for 48 hours followed by Epo for 48 hours. Fifty-eight percent of the cells were Epo-specific Hb+. This contrasts with unprimed cells in which only 2% were Hb+ after 2 days of Epo. In addition, the maximal Epo response was seen earlier in the primed cells. To demonstrate this increase in the rate of Epo response, in Fig 4B we have replotted the data from Fig 4A and normalized them to percent of maximal Epo-specific response. A pronounced shift in the maximal Epo response time from 4 days in unprimed to 2 days in DMSO-primed cells is observed.

**DMSO priming markedly increases Epo sensitivity.** We examined the Epo dose-response relationships in DMSO-primed and unprimed cells. As presented in Fig 5, there is a marked left-shift of the dose-response curve. The Epo activation constant (K_α), defined as the concentration of agonist that causes 50% of the maximal response, is 0.1 U/mL and 2 U/mL for DMSO-primed and unprimed cells, respectively, representing an increase in sensitivity of approximately 20-fold. The length of Epo exposure had no significant effect in the K_α, in either cell group under each condition tested (data not shown).

**Epo priming does not affect the DMSO response.** The striking effects of DMSO priming on the Epo response led us to ask whether the converse was true, ie, did "Epo priming" amplify the DMSO response? Cells were incubated in the absence or presence of 10 U Epo/mL for 24 hours. The cells were washed and plated in the presence of 1% DMSO. The percent of Hb+ cells in each culture was determined after an additional 16 hours and 48 hours of growth in DMSO. The unprimed cultures contained 0% and 23% ± 3% Hb+ cells, whereas the Epo-primed cultures contained 3% ± 2% and

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**Fig 3.** DMSO priming of the Epo response is time-dependent (A) and concentration-dependent (B). (A) Cells were grown in 1% DMSO for the times indicated. Cells were washed twice with 3% FBS/DMEM to remove DMSO and replaced with in fresh medium containing Epo (10 U/mL). (B) Cells were pretreated with DMSO at the indicated concentrations for 24 hours, washed twice with 3% FBS/DMEM to remove DMSO, and replated in fresh medium with Epo (10 U/mL). The percentage of Hb+ cells was measured after 24 hours of treatment with Epo.

**Fig 4.** DMSO priming increases number of Epo-responsive cells (A) and rate of Epo response (B). (A) Cells were grown in the absence (○) or presence (●) of 1% DMSO for 48 hours, washed twice with 3% FBS/DMEM, and exposed to Epo (40 U/mL) beginning on day 0. Net Epo specific Hb+ response was obtained by subtracting the basal Hb+ cell number at the times indicated. (B) The same set of data in (A) was normalized to percent of maximal Hb+ response.

**Fig 5.** DMSO priming markedly increases Epo sensitivity of Rauscher cells. Cells were grown in the absence (○) or presence (●) 1% DMSO for 48 hours, washed twice with 3% FBS/DMEM, and replated in the presence of specific concentration of Epo for 24 hours. Net Epo response was obtained by subtracting the basal Hb+ cell number (no addition of Epo) from the total Hb+ cell number at various Epo concentrations. Data were normalized using the percent of maximal Epo-specific response (obtained at 10 U/mL). The percentage of Hb+ cells induced by Epo at 10 U/mL was 10% and 41% for unprimed cells and primed cells, respectively. Inset: Horizontal scale enlarged to facilitate comparison at low Epo concentrations.
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26% ± 3% Hb+ cells after 16 hours and 48 hours of DMSO treatment, respectively. We conclude that Epo priming had no significant effect on the DMSO response.

**DMSO increases the number of Epo receptors on Rauscher cells.** The multiple effects of DMSO priming on the Epo biologic response in Rauscher cells are all consistent with an amplification of the Epo signal transduction pathway by the chemical inducer. To test this possibility, we examined the effect of DMSO priming on the site of initiation of the signal, i.e., the Epo receptor itself.

The binding of 125I-Epo to unprimed Rauscher cells was specific and saturable (Fig 6, open circles). A Scatchard analysis of these binding data showed two populations of receptors with equilibrium dissociation constants (kd) of 1.1 nmol/L and 6.2 nmol/L, respectively (Fig 7). The results indicate approximately 1,200 higher affinity receptors and 2,600 lower affinity receptors, for a total receptor density of approximately 3,800 per cell. The results of 125I-Epo binding to DMSO-primed Rauscher cells were strikingly different (Fig 6, closed circles). At lower 125I-Epo concentrations, the binding curve was virtually identical to that obtained with unprimed cells (Fig 6, open circles), a finding that was confirmed by us repeatedly. As expected, the Scatchard analysis of this portion of the curve (not shown) was virtually identical to the higher affinity population seen in Fig 7 (kd = 1.0 nmol/L; 1,100 receptors/cell). However, at higher 125I-Epo concentrations, a new high density receptor population was detected, reaching near saturation at ~20,000 receptors/cell (Fig 6). The Scatchard analysis of 125I-Epo binding to these new receptors found on DMSO-primed cells showed a pronounced upwardly convex geometry (Fig 8). This result, which was obtained each time in three separate experiments using both this R28 Rauscher cell line as well as another, independently derived line, is considered to be diagnostic of positive cooperativity among receptors.16

**DISCUSSION**

DMSO is known to be a powerful inducer in several different systems, including erythroid and myeloid differentiation.1,17 DMSO-induced differentiation of murine erythroleukemia cells in vitro has facilitated characterization of the terminal differentiation process. An alteration in the rate of calcium transport appears to be the rate-limiting event for DMSO-induced commitment of murine erythroleukemia (MEL) cells.18 Faletto et al19 have found that levels of the phosphatidylinositol metabolites inositol-triphosphate and diacylglycerol decrease significantly within 2 hours of DMSO-induced differentiation of Friend cells. Thus, diacylglycerol regulation of kinase C activity may play a key role in control of DMSO-induced differentiation. Balazovich et al20 also suggested that changes in protein kinase C activity and distribution are associated with DMSO-induced Friend cell differentiation. The signaling pathway underlying the action of Epo is unclear. Alterations in Ca2+ metabolism by Epo were observed in Friend virus-infected erythroid cells21 and early human erythroid precursors,22 but not in rabbit erythroblasts23 or phenylhydrazine-treated mice spleen cells.24

The present studies demonstrate that there is interaction of the signaling pathways of DMSO and Epo in Rauscher erythroleukemia cells. Pretreating the cells with DMSO potentiates their response to Epo in three ways. Firstly, the total percentage of Epo responsive cells is increased by DMSO. According to the stochastic model of in vitro differentiation of erythroleukemia cells proposed by Gusella

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**Fig 6.** 125I-Epo binding to Rauscher cells grown in the absence (○) or presence (●) of 1% DMSO for 24 hours. Points are means of triplicate samples. Specific binding is shown. Nonspecific binding was less than 10% of total. Similar results were obtained in two other experiments. See Materials and Methods.

**Fig 7.** Scatchard analysis of 125I-Epo binding to Rauscher cells grown in the absence of DMSO (unprimed) (○ in Fig 6).

**Fig 8.** Scatchard analysis of 125I-Epo binding to new receptors on DMSO-primed Rauscher cells (● of Fig 6).
et al., 25 uncommitted cells have a probability (P) of becoming committed and a probability (1 - P) of remaining uncommitted during any given cell generation. Our results show that DMSO priming increases the probability (P) of Rauscher cells becoming committed in response to Epo. Secondly, DMSO priming increases the rate of response to Epo, i.e., the time required for appearance of maximum numbers of Hb+ cells is reduced from 4 days to 2 days. Thirdly, DMSO priming increases the Epo sensitivity of Rauscher cells approximately 20-fold as manifested by a pronounced leftward shift of the Epo dose-response curve.

A modification of the Epo response by DMSO in a novel MEL cell line has been reported previously. 26 Addition of DMSO to TSA8 cells allowed the cells to form colonies on Epo stimulation. These investigators suggested that DMSO converted TSA8 cells into a later stage of differentiation similar to that of the colony-forming unit-erythroid (CFU-E). In this regard, the amplified biologic response we have observed in DMSO-primed Rauscher cells, most notably the 2-day maximal response rate and the dose-response curve, is also remarkably similar to the normal murine CFU-E. In contrast to the up-regulation of Rauscher cell Epo receptor that we have observed, DMSO apparently had little or no effect in TSA8 cells. 27 DMSO treatment of another murine erythroleukemia Rauscher cell line has been reported to increase Epo receptor density approximately fivefold. 28 No effect of this DMSO treatment on the Epo biologic response of these cells was reported.

Effects of DMSO on the actions of other inducers have been found in several different systems. In a recent report of Schwartz and Maher, 29 DMSO induced differentiation of a promyelocytic leukemia cell line, HL60, and increased the number of high affinity granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors. In 3T3 cells, DMSO did not induce any apparent morphologic change. However, it changed the sensitivity of these cells to the activation of Na+/H+ exchanger by protein kinase C activators. 30 In Friend cells, suboptimal amounts of DMSO were reported to prime cells for rapid commitment in response to further contact with the same or even a different chemical inducer. 31 However, we found no such effect of DMSO priming on further DMSO-induced differentiation in Rauscher cells. Pretreating Rauscher cells with 1% DMSO for either 1 or 2 days did not enhance their response to freshly added DMSO (data not shown), suggesting that DMSO priming of Rauscher cells appears to be specific for the Epo signaling pathway.

We have reported previously that the pathways of Epo- and DMSO-induced differentiation are dissimilar. 26 Because the ultimate result of the two inducers is hemoglobinization, the two signal pathways may intersect at some point downstream, resulting in a series of "common pathway" events. If DMSO potentiates the Epo response at these hypothetical common pathway events, then one might expect that Epo pretreatment would likewise augment the DMSO response by facilitating these same events. However, we found that Epo pretreatment does not amplify the DMSO response, suggesting that facilitation of events common to both inducers is not the means by which DMSO amplifies Epo's action.

We have identified one effect of DMSO priming that may explain, at least in part, the amplified Epo response. DMSO treatment resulted in the appearance of a new population of Rauscher receptors of relatively high density (\(\sim 20,000/\text{cell}\)). The Scatchard analysis of \(^{125}\text{I}\)-Epo binding to these receptors shows marked positive cooperativity. 32 Although seen commonly with steroid hormone receptors, 33 positive cooperativity has been reported only rarely with polypeptide hormone receptors 33, 34 and apparently has not been observed previously with the Epo receptor. Because of this property, an equilibrium dissociation constant cannot be calculated for this population. We speculate that this finding may reflect protein-protein interaction among receptors, possibly involving accessory molecules. This association of receptors could facilitate a more pronounced Epo signal. Further studies to elucidate the molecular basis for this receptor up-regulation are in progress.

Interestingly, at least three other hematopoietic growth factors (BPA, interleukin-3 [IL-3], and GM-CSF) have been reported to be involved in the process of erythropoiesis. 35-37 Using FBS-supplemented cultures of progenitor cells from adult human marrow, IL-3 was reported to increase the number of burst-forming unit-erythroid (BFU-E)-derived colonies induced by Epo, while GM-CSF did not increase the number of colonies but did increase the sensitivity of BFU-E to Epo. 37 Thus, DMSO may mimic the functions of other growth factors that interact with Epo. It will be of importance to analyze DMSO's action further with regard to the modes of action of these natural inducers. Such comparisons may prove fruitful in efforts directed toward the design of therapeutic agents that may be used in conjunction with recombinant growth factors. Moreover, DMSO and other chemical agents may be considered as convenient probes of Epo's signal transduction pathway.

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