The Use of Mice Made Tolerant to Rat Red Blood Cells in Erythropoietin Assays (36007)

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The most sensitive assays available for standardization of erythropoietin (EP) (1) involve the use of mice made polycythemic by transfusion of isologous red blood cells (2), or by exposure to environments with a low partial pressure of O_2 (3). The former method requires a large number of donors, is tedious and time consuming; the latter requires a low-pressure tank. It was thought, that for sporadic use in clinical laboratories, it might be useful to have a method of making mice polycythemic, which did not require a pressure vessel, or the use of a large number of blood donor mice. The recent findings that mice can be made tolerant to heterologous cells (4) by cyclophosphamide injection and that stem cells recover quickly after tolerizing doses of this drug (5), suggested the possibility of using heterologous red blood cells for inducing polycythemia in tolerant mice. Rat RBC were chosen instead of sheep RBC, as mice do not have natural antibodies to rat RBC (6), and rat RBC remain longer in the circulation of untreated mice than do sheep RBC (7).

Materials and Methods. Female Balb/c mice, 10-12 weeks old, were used as recipients and adult Canberra Black rats as blood donors. Tolerance was induced by intravenous injection of 1 ml 50% v/v suspension of thrice saline-washed rat RBC, followed 1 day later by an intraperitoneal injection of 250 mg/kg of cyclophosphamide (CP) (Endoxan Asta).

In preliminary experiments it was observed that 1 week after transfusion of ^{51}Cr-labeled rat RBC, 60 ± 16% of the ^{51}Cr was in the circulating blood in the cyclophosphamide-treated animals, which had a packed-cell volume (PCV) of 57 ± 2%, while none was detectable in the noncyclophosphamide-treated animals, PCV 48 ± 1.25. When a second intravenous injection of 1 ml 50% rat RBC was given after sampling for ^{51}Cr, the cyclophosphamide-treated group showed a PCV of 71% the next day, while all the non-CP-treated animals were dead. A week after the second transfusion the PCV in the CP-treated animals was 59 ± 2%.

On the basis of these findings and the data on recovery of marrow stem cells after cyclophosphamide injection (5), the treatment regimens outlined in Table I were tried for EP assay. The erythropoietin preparation used was the International Reference Preparation, kindly supplied by Dr. Mary Cotes, Division of Biological Standards, National Institute for Medical Research, London. EP was injected subcutaneously. ^{59}Fe citrate (Radiochemical Centre, Amersham, U.K.) was injected intravenously in a dose of 0.2 μCi/mouse. Radioactivity of RBC from 0.2 ml of blood, washed once in 10 ml of saline, was measured in a Packard Autogamma scin-

TABLE I. Treatment Regimens.

	Treatment	1 ml RRBC	CP,[a] 250 mg/kg	1 ml RRBC	1 ml RRBC	PCV EP	^{59}Fe	Bleed PCV
Group A	Day	0	1	5	8	10	12	15
Group B	Day	0	1	5	10	12	14	17

[a] Cyclophosphamide.

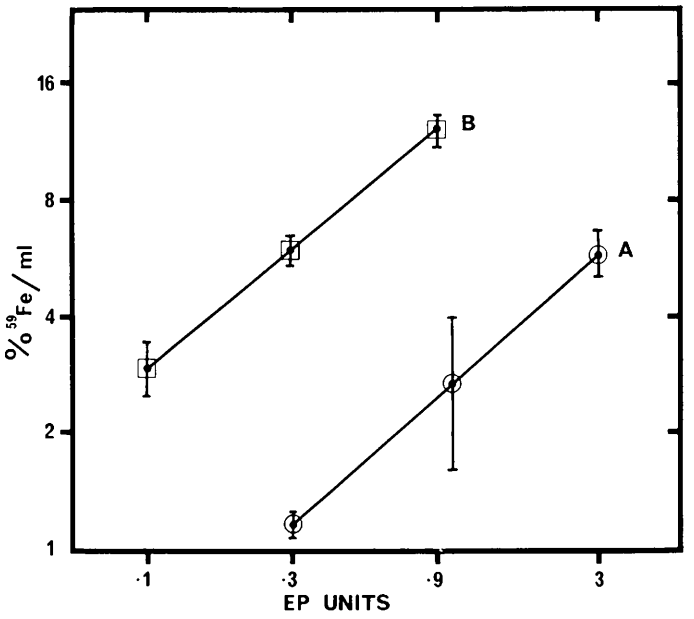

FIG. 1. Percentage of ^{59}Fe in RBC. Ordinate: (log scale). Dose of erythropoietin (EP) abcissa: (log scale). EP injected 9 days line A; and 11 days, line B, after cyclophosphamide injection into rat RBC-transfused mice.

tillation counter.

Results. Of 220 rat RBC-transfused mice injected with cyclophosphamide only seven died. The survivors were all polycythemic. Figure 1 shows two log dose–log response lines, obtained when EP was injected 9(A) and 11(B) days after a single injection of cyclophosphamide. The statistical analysis of the regressions are shown in Table II. Both lines are parallel with line B shifted 1 log unit to the left of A. The results of other assays are shown in Table III, when EP injection was delayed until 13 days after cyclophosphamide injection, no significant increase in sensitivity was observed. PCV, measured with a microhematoent centrifuge in blood obtained from the retroorbital sinus of 26 mice before EP injection was 70% (range 63–83%) and in heart blood obtained 3 days after ^{59}Fe injection 60% (56–64).

Discussion. There is a statistically significant linear relationship between log dose of EP and log response of tolerant mice made polycythemic by transfusion of rat RBC. Maximum sensitivity was obtained when EP was injected 11 days after cyclophosphamide,

TABLE II. Regression Analysis of Log Response % ^{59}Fe versus Log Dose of Erythropoietin Carried Out Using the COLNR$ Program and the G. E. 265 Time-Sharing Computer.

	Line A			Line B		
	D. F.	Sum of squares	Mean square	D. F.	Sum of squares	Mean square
Source of variation total	15	1.9099	.1273	21	1.7439	.083043
Regression	1	1.5704	1.5704	1	1.3575	1.3575
Error	14	.3396	.02426	20	.3864	.01932
Index of determination		.8222			.7784	
Correlation coefficient		.9068			.8823	
F-Ratio test statistic		64.7415	$p < .001$		70.2727	$p < .001$
Slope		$.709 \pm 0.096$			$.693 \pm 0.080$	

TABLE III. Response (% ^{59}Fe/ml ± S.E.) to Erythropoietin of Rat RBC-Transfused Mice Injected at 9, 11, and 13 Days After Cyclophosphamide.

Dose (U)	Day of injection[a]			
	9	11	11	13
1.0	—	—	14.4 ± 2.8 (3)	—
0.3	1.16 ± 0.07 (8)	9.7 ± 0.85 (9)	8.6 ± 1.17 (4)	10.2 ± 0.9 (9)
0.1	0.06 ± 0.01 (8)	2.4 ± 0.35 (9)	2.8 ± 0.31 (4)	3.5 ± 0.17 (9)
0.05	—	—	—	1.2 ± 0.19 (9)
Saline	0.06 ± 0.02 (8)	0.24 ± 0.03 (7)	0.51 ± 0.04 (4)	0.12 ± 0.04 (5)

[a] Number of mice indicated in parentheses.

the sensitivity was much less when EP was injected at 9 days. One unit injected at 9 days gave the same response as 0.1 U injected at 11 days. This indicates very fast recovery of EP-response cells between days 9 and 11 after cyclophosphamide injection.

The sensitivity and precision of this assay compares well with other polycythemic mouse assays (1). The assay requires few donor rats, is simple to carry out, and could prove practical for laboratories not equipped with pressure tanks.

Summary. Mice were made tolerant to rat red blood cells, by the injection of 250 mg/kg of cyclophosphamide. Such tolerant mice, made polycythemic by three transfusions of 1 ml of 50% rat RBC suspension, show a good response to erythropoietin injected 11 days after cyclophosphamide and may be used in the assay of erythropoietin.

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