FACTORS INFLUENCING HEMATOPOIETIC SPLEEN COLONY FORMATION IN IRRADIATED MICE

II. THE EFFECT OF FOREIGN MATERIALS*

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Irradiated mice form macroscopic nodules on the spleen which are composed of hematopoietic tissue. The preceding report (1) was concerned with elucidating certain characteristics of endogenous spleen colony formation in normal irradiated mice. This report is concerned with changes in endogenous colony formation induced by the injection of mice with foreign plasma and with other materials. A word of explanation is offered concerning our reasons for electing to test the effect of foreign plasma upon this system.

We began studies of endogenous spleen colony formation because we anticipated that this system might prove useful for studying factors concerned with the humoral regulation of hematopoiesis. Preliminary studies by others (2–5) using this and similar systems for this purpose have been reported. We previously reported (6, 7) that the plasma of dogs, recovering from neutropenia induced with vinblastine sulfate, nitrogen mustard, and endotoxin, will accelerate the rate of release of neutrophils from bone marrow to blood of normal dogs. By utilizing the endogenous spleen colony system we hoped to be able to determine whether this neutrophil-releasing factor was capable of directly stimulating granulocyte production. However, this objective was delayed when pilot studies disclosed that normal dog plasma, which has no neutrophil releasing effect in the dog, profoundly affected spleen colonies in the mouse.

The data reported herein relate to the effect on hematopoietic spleen colonies by preirradiation injection of mice with normal, foreign plasma as well as with other substances. The results suggest that a change in spleen colony formation

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resulting from injected substances (2, 3) need not constitute evidence that the injected substance can be considered a homeostatic regulator of hematopoiesis.

Preliminary reports of a part of these studies have appeared (8, 9).

Materials and Methods

Detailed methodology for the study of endogenous spleen colonies is included in the preceding companion report (1).

The mice were F_1(C57BL/6J ♀ × DBA/2J ♂) and were 7–12 wk of age when killed. All studies of endogenous spleen colony formation were done 10 days after irradiation.

Blood for determination of total leukocyte count and volume of packed red cells (VPRC) was collected from the orbital sinus in heparinized microhematocrit tubes. Leukocyte counts were performed electronically by a slight modification (10) of the technic of Gagon et al. (11). Differential leukocyte counts of 200 cells were performed on Wright's stained coverslips of orbital sinus blood. The method used for measuring the total number of nucleated marrow cells in a mouse humerus has been reported in detail (10).

Acid-citrate-dextrose (ACD-B, Cutter Laboratories, Berkeley, Calif.) was the anticoagulant used in collecting whole blood from which plasma was separated. All blood collection and plasma separation was carried out in pyrogen-free plastic and glassware. Plasma was frozen at −10°C until immediately before use.

The sheep red cells used for injection were washed six times with pyrogen-free saline and injected as an 80% suspension. Mumps and influenza vaccines (Eli Lilly and Co., Indianapolis, Ind.) were injected undiluted. Phytohaemagglutinin M (Difco Laboratories, Inc., Detroit, Mich.) was dissolved in pyrogen-free saline, 5 ml per vial. Human fibrinogen (Parke, Davis & Co., Detroit, Mich.) was dissolved in pyrogen-free saline at a concentration of 23 mg/ml. Human gamma globulin (poliomyelitis immune globulin, Cutter Laboratories), 16 mg/ml, human albumin (Hyland Laboratories, Los Angeles, Calif.), 250 mg/ml, and hydrocortisone phosphate (Merck, Sharp & Dohme, West Point, Pa.), 20 mg/ml, were injected undiluted. Endotoxin (Salmonella typhosa, lot 474995, Difco Laboratories) was diluted with pyrogen-free saline to the desired concentration and injected intraperitoneally at a constant volume of 0.5 ml.

Mice were hypertransfused by injecting isogeneic or C57BL (maternal) erythrocytes intraperitoneally before irradiation (1). A volume of packed red cells of 60%, 10 days after irradiation at the time when mice were killed, was required before the mice were considered plethoric.

In certain instances, results of various experiments have been combined in tables. In each experiment an irradiated, but otherwise untreated, control group was included. In tables in which experiments were combined, results are expressed as a ratio of the experimental group to the irradiated control group.

RESULTS

Effect of Normal Dog Plasma.—Injection of pooled, normal dog plasma, 0.5 ml intraperitoneally per day for 4 days preceding irradiation, resulted in an increase in the number of colonies per spleen, spleen weight, splenic iron-59 (^{59}Fe) uptake, and splenic ^{125}I iododeoxyuridine (IUdR) uptake at 10 days after irradiation (Table I). Injection of saline or acid-citrate-dextrose, the anticoagulant in the plasma, was without effect (Table I).

This effect upon splenic hematopoiesis was evident with as much as 1000 R irradiation exposure (Fig. 1). At all levels of irradiation exposure studied,

colonies were increased approximately 10-fold in plasma-injected animals when compared to irradiated controls (Fig. 1). The D_o (the irradiation required to reduce colonies to 37 % of original) was similar for control and plasma-injected animals (Fig. 1).

In order to determine the interrelationship between spleen weight, ^{59}Fe uptake, and colony number, data from all mice injected with plasma for 4 days preceding irradiation, independent of the irradiation exposure, were pooled and analyzed utilizing a Control Data 3200 computer. Splenic ^{59}Fe uptake increased in direct relationship to the number of colonies present in spleens from plasma-injected mice, and the approximate rate of increase was

TABLE I

*The Effect of Injecting Normal Dog Plasma upon Splenic Hematopoiesis in Irradiated Mice**

	Mice per group	Spleen weight	Iron	IUdR	No. of colonies
			% of unirradiated control		
		mg			
Control Groups					
No injection	20	21 ± 1‡	20 ± 2‡	55 ± 4‡	3.2 ± 0.3‡
Saline	10	17 ± 2	15 ± 2	—	2.8 ± 0.6
ACD	10	22 ± 2	23 ± 2	—	2.9 ± 0.5
Plasma-injected	20	50 ± 2	160 ± 5	193 ± 10	28.4 ± 3.1

* Mice killed 10 days after exposure to 700 R. Dog plasma, 0.5 ml, was injected for 4 consecutive days preceding irradiation. Saline or saline with 12% acid-citrate-dextrose (ACD), the maximum concentration of ACD in plasma, were injected in the same dosage and on the same schedule as the plasma.

‡ Mean value ± standard error.

not significantly different from that observed in noninjected mice (Fig. 2 a). Spleen weight increased at an approximate rate of 1.2 mg per colony, a relationship not significantly different from that observed in uninjected mice (Fig. 2 b). Spleens without colonies from injected mice weighed more than those from uninjected mice (22 mg compared to 18 mg, $P = < 0.05$). The relationship between spleen weight and iron uptake was that predicted on the basis of their mutual relationship to the number of spleen colonies (Fig. 3). However, at spleen weights above 60 mg, with confluent (in excess of 30) spleen colonies, this relationship disappeared (Fig. 3). Furthermore, there was no detectable relationship between IUdR uptake and spleen weight in spleens with confluent colonies weighing more than 60 mg (data not shown).

No untoward effects of plasma administration were noted. There was no significant difference in body weight of injected or uninjected mice 10 days after irradiation.

Dose Response and Time Relationship of Plasma Injection to Splenic Hematopoiesis.—In mice injected with 0.1 ml of plasma per day for 4 days before irradiation, a slight but significantly increased number of spleen colonies was noted (Table II). Increasing the dose to 0.25 and to 0.50 ml per day each resulted in an increment in the magnitude of effect (Table II).
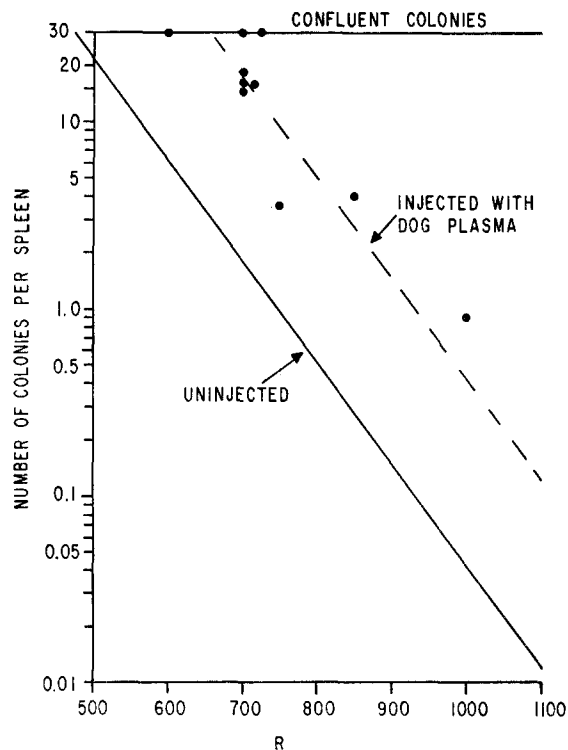

Fig. 1. Relationship of irradiation exposure to number of spleen colonies in mice injected with dog plasma for 4 days preceding irradiation. Each point represents the average number of colonies in a group of 10 or more mice. The line describing this relationship in mice which were not injected with plasma (1) is included for comparison.

The most sensitive index of a statistically significant change in splenic hematopoiesis was colony number, followed by iron uptake and spleen weight.

The route of administration influenced the magnitude of plasma effect (Table II). Intravenous administration proved most effective. Subcutaneous administration, though effective, was less so than intraperitoneal administration.

A single injection of 0.5 ml of plasma on any 1 of 4 days preceding irradiation and an injection on both day 1 and 2 before irradiation were ineffective (lines
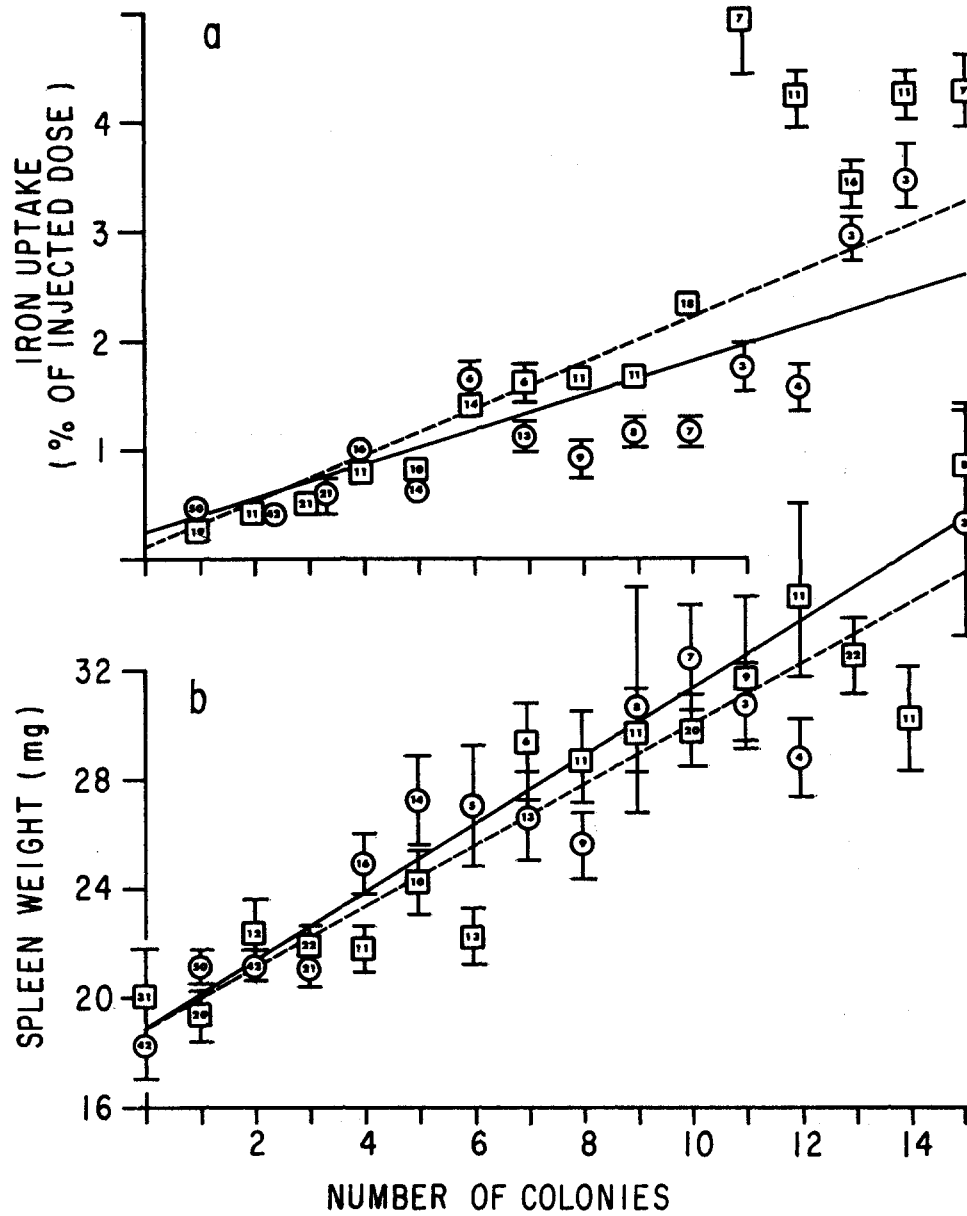
FIG. 2. Relationship of uptake of radioactive iron by the spleen and of spleen weight to number of colonies in mice injected with dog plasma for 4 days preceding irradiation. Each point represents the mean value from groups of 3 to 50 mice and the number making up each point is noted in the figure. The squares and the dashed line represent plasma-injected mice and the circles and solid line, uninjected mice. The standard error for each point is shown.

4–8, Table III). The total volume injected in any of these groups equals or exceeds that of the effective 4 day schedule of 0.1 ml/day in Table II, line 2, indicating that the repetitive dose schedule has a greater influence on the effectiveness of plasma than does the total dose. However, a single dose given 1 hr before irradiation had a slight but significant effect (Table III, line 1).
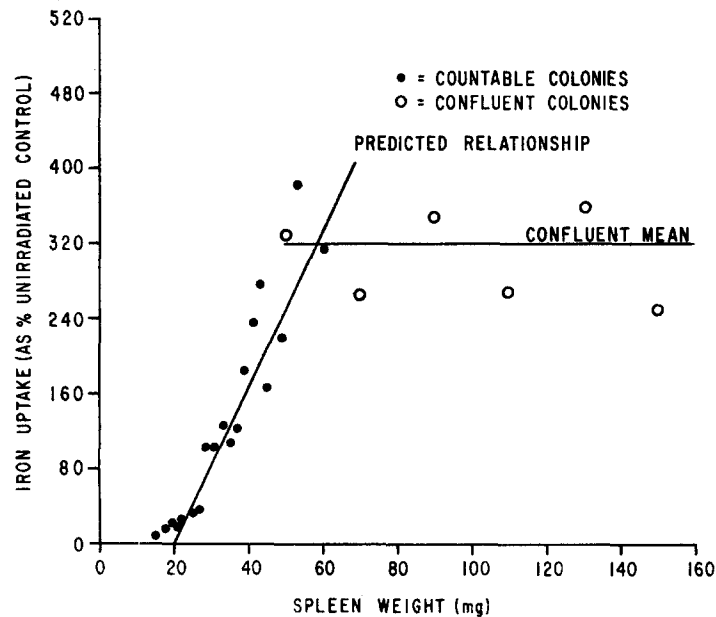

Fig. 3. Relationship of uptake of radioactive iron by the spleen to spleen weight in mice injected with dog plasma for 4 days preceding irradiation. In groups of animals in which countable numbers of spleen colonies were present (solid dots) the line describing this relationship which was predicted from the relationship of each of these values to the number of colonies (see Fig. 2) appeared to be valid. However, in spleens with confluent colonies (open circles) there was no apparent relationship between spleen weight and iron uptake. The open circles represent groups of 15 or more mice with confluent colonies grouped according to spleen weight of less than 60 mg, 60–79 mg, 80–99 mg, 100–119 mg, 120–139 mg, and more than 140 mg.

An increasing effect was obtained by increasing the number of daily injections from three to four to five before irradiation (lines 9–12) but 6 and 8 days of administration (lines 13–14) were no more effective than 5 days (lines 11 and 12, Table III).

Increasing the interval between the last of five consecutive injections and the time of irradiation exposure from 1 hr to 1 day did not change the effectiveness of the 5 day dosage schedule (line 11 and 12, Table III). However, if the last of a series of four injections was given 3 days before irradiation, the

effectiveness of the schedule was reduced (line 15 vs. line 10) and, if the last injection was given 5 days before irradiation, the effect was virtually abolished (line 16). Spacing injections 1 wk apart was an ineffective schedule (line 17).

One injection of 0.5 ml of plasma given 1 hr after irradiation was ineffective (line 2, Table III) but if 1.0 ml of plasma was given at this time a slight but significant effect was observed (line 3). No other effective dosage schedules were found for postirradiation plasma injections, although up to nine consecutive daily injections of plasma from six different dogs were studied (line 18).

TABLE II

The Effect of Different Volumes of Dog Plasma and Different Routes of Administration of Plasma Upon Splenic Hematopoiesis in Irradiated Mice

Route of injection*	Volume injected per day	Radiation exposure	Splenic hematopoiesis		
			Weight	Iron (% of unirradiated control)	No. of colonies
	ml	R	mg		
Control	—	750	21 ± 1‡	7 ± 1‡	9.8 ± 0.3‡
Intraperitoneally	0.10	750	21 ± 1	9 ± 2	1.7 ± 0.8§
"	0.25	750	20 ± 1	13 ± 3	2.3 ± 0.6
"	0.50	750	23 ± 1	26 ± 7	3.5 ± 0.6
Control	—	700	20 ± 1	6 ± 1	2.3 ± 0.4
Subcutaneously	0.25	700	26 ± 2	26 ± 4	8.6 ± 2.1
Intraperitoneally	0.25	700	30 ± 3	53 ± 13	14.7 ± 2.1
Intravenously	0.25	700	40 ± 4	192 ± 18	20.0 ± 9.1

* Given for 4 consecutive days before irradiation.

‡ Mean ± standard error for group of 10 mice.

§ Differs from control by P of less than 0.05 by t test if value is underlined.

The Effect of Plasma Injections upon Hypertransfused Mice.—Mice made plethoric by preirradiation transfusion have a reduced number of macroscopic spleen colonies and a splenic iron-59 uptake indistinguishable from that of mice in which spleens are without colonies (1). The residual colonies in plethoric mice are quite small and are composed primarily of granulocytes (1, 12). The similarity of the slope of the colony splenic iron-59 uptake curve of plasma-injected mice (Fig. 2 a) to that for uninjected mice (1) suggested that all hematopoietic elements in the spleen were proportionally increased by 4 days of dog plasma injections preceding irradiation. To further study this question, mice were hypertransfused before irradiation and the effect of plasma injection upon nonerythroid colonies was studied.

In plethoric, plasma-injected mice significantly more colonies were observed than in plethoric mice without plasma injection (line 2, Table IV) ($P = < 0.01$).

The increase observed is of the same order of magnitude as that seen in non-plethoric mice. However, there was no difference between plasma-injected and noninjected plethoric mice with respect to splenic iron-59 uptake and ^{125}I-UdR uptake (Table IV). Histologic examination revealed no erythroid colonies in plethoric mice but there was an increase in granulocytic and megakaryocytic colonies in the spleens of plasma-injected plethoric mice.

TABLE III

Relationship of Time of Injection and Number of Injections to the Effect of Plasma Upon Splenic Hematopoiesis

Line	Day of plasma injection in relation to day of irradiation (day 0) (0.5 ml/injection)	Mice per group	Splenic hematopoiesis			
			Spleen weight	IUdR	Iron	Colonies
			(Ratio of experimental group to control)			
1	0: 1 hr before	30	1.0		1.2	1.6*
2	1 hr after	20	0.9		0.9	1.0
3	1 hr after, 1.0 ml	20	1.4		2.2	1.7
4	−1	20	1.2		1.5	0.9
5	−2	10	1.0		1.2	1.0
6	−3	10	1.1		1.0	0.4
7	−4	10	1.1		1.5	0.7
8	−1, −2	10	0.9		1.1	1.2
9	−1, −2, −3	10	1.0		1.3	2.1
10	−1 through −4	60	2.0	4.0	8.3	10.2
11	0 through −4	20	4.1		18.2	>10.0
12	−1 through −5	10	5.8	8.7	18.7	>10.0
13	−1 through −6	10	6.1	11.9	16.7	>10.0
14	−1 through −8	20	4.8	10.6	19.1	>10.0
15	−3 through −6	20	1.9		9.0	4.3
16	−5 through −8	20	1.4		1.3	1.3
17	−7, −14, −21	20	1.2		0.9	0.6
18	+1 through +9	60	1.0		1.1	0.7

* Differs from matched control group(s) by P of <0.05 by t test if value is underlined.

Effect of Plasma from Different Dogs upon Splenic Hematopoiesis.—Except where otherwise specified, all studies of dog plasma involved the use of a single batch of plasma obtained by exsanguinating four large dogs and pooling their plasma. To be certain that the effect observed with this plasma was not peculiar to that plasma, the effect of plasma from seven different normal dogs was studied. An increase in splenic hematopoiesis accompanied injection of each of the plasmas (Table V). The magnitude of effect differed significantly between different plasmas. To further explore the possibility that the difference between plasmas was larger than could be explained by chance, a second experiment was performed (lines 9–15, Table V). From two dogs, three plasma

TABLE IV

The Effect of Plasma Injection Upon Splenic Hematopoiesis in Plethoric, Irradiated Mice*

Plethoric mice	Mice per group	Splenic hematopoiesis			
		Spleen weight	Iron	IUdR	No. of colonies
			(% of unirradiated control)		
Controls	30	*mg* 25 ± 2‡	9 ± 1‡	42 ± 4‡	0.9 ± 0.2‡
Plasma-injected	35	28 ± 2	9 ± 1	40 ± 3	7.6 ± 1.2

* See Materials and Methods for details of transfusion. Normal dog plasma, 0.5 ml, was injected intraperitoneally for 4 days before irradiation. The effect of plasma in plethoric mice was compared at radiation exposures of 650, 700, and 750 R. Since no significant difference was observed in the effect of plasma at the three exposure levels, results are combined in the table.

‡ Mean ± standard error.

TABLE V

Comparison of the Effect of Plasma From Different Dogs Upon Splenic Hematopoiesis in Irradiated Mice*

Line	Dog	Splenic hematopoiesis		
		Spleen weight	Iron (% of unirradiated control)	Colonies (number per spleen)
1	Control	*mg* 22 ± 2‡	35 ± 6‡	2.9 ± 1.0‡
2	1-81	107 ± 18	450 ± 53	>30§
3	1-31	106 ± 20	482 ± 80	28.3 ± 5.0
4	2-77	55 ± 9	266 ± 75	22.0 ± 5.1
5	2-79	92 ± 10	377 ± 65	>30§
6	2-80	123 ± 13	422 ± 53	>30§
7	2-81	91 ± 15	234 ± 44	26.0 ± 5.6
8	1-79	49 ± 15	211 ± 47	23.6 ± 5.6
9	Control	17 ± 1	11 ± 2	1.0 ± 0.3
10	1-79-1	30 ± 4	113 ± 52	9.4 ± 2.4
11	-2	29 ± 2	62 ± 19	6.8 ± 2.2
12	-3	25 ± 2	36 ± 17	3.8 ± 2.4
13	1-59-1	37 ± 1	145 ± 41	11.7 ± 2.0
14	-2	32 ± 5	104 ± 43	10.7 ± 4.8
15	-3	40 ± 5	213 ± 75	14.1 ± 4.2

* Plasma, 0.5 ml, injected intraperitoneally for 4 consecutive days before irradiation (700 R in experiment shown in lines 1-8, 750 R in lines 9-15). Irradiated control groups (lines 1 and 9) with 10 mice per group, all others with 5 mice per group.

‡ Mean of group ± standard error.

§ Confluent, uncounted colonies in all spleens in group. A value of 40 was assumed for confluent spleens in other groups.

samples were collected at $\frac{1}{2}$ hr intervals and tested. Plasma from dog 1-59 was consistently more effective than that from dog 1-79 ($P = < 0.05$).

The Effect of Previous Plasma Injections and Irradiation upon Subsequent Response to Plasma Injection.—Two groups of 20 mice were irradiated. One group was injected with plasma before irradiation. Each was allowed to recover and, 7 wk after irradiation, was again irradiated. One-half of each of the original groups was given plasma before the second irradiation. Mice which were injected with plasma before the first, but not before the second, irradiation had larger spleens ($P = < 0.05$) than those which never received plasma

TABLE VI

The Influence of Previous Exposure to Plasma Injection and Irradiation on the Effect of Plasma Injection Upon Splenic Hematopoiesis*

| First irradiation | Second irradiation | Splenic hematopoiesis 10 days after second irradiation | | |
		Spleen weight	Iron uptake (% of unirradiated control)	No. of colonies
		mg		
700 R	725 R	20 ± 1‡	32 ± 13‡	3.9 ± 1.1‡
700 R	725 R + plasma	107 ± 12	420 ± 39	>30§
700 R + plasma	725 R	27 ± 2	36 ± 8	4.9 ± 1.4
700 R + plasma	725 R + plasma	119 ± 12	482 ± 48	>30§

* Normal dog plasma, 0.5 ml, injected intraperitoneally for 4 consecutive days preceding either first or second irradiation or both.

‡ Mean of group of 8 to 10 mice ± standard error.

§ All spleens in group with confluent spleen colonies.

(Table VI, line 3 vs. line 1). However, there was no significant difference in response to the second irradiation with respect to colony number or iron-59 uptake (Table VI). The magnitude of the increase in splenic hematopoiesis, induced by plasma injection before the second irradiation, was not influenced by prior plasma injection (Table VI, line 4 vs. line 2).

The Effect of Plasma Injections upon Nonsplenic Measures of Hematopoiesit in Irradiated Mice.—10 days after irradiation, the number of nucleated cells per marrow cavity of the humerus was slightly but not significantly ($P = > 0.02$) higher in plasma-injected than in saline-injected mice (Table VII). Smears made from these marrows contained very few lymphocytes and more than 90% of the marrow cells were identifiable as granulocytes, erythroblasts, or megakaryocytes. Differential counts were not performed since the marrow cavity appeared to be regenerating in a colonial fashion and smears tended to be nonuniform in cellular proportions between the three main cell types.

The volume of packed red blood cells (VPRC) was higher ($P = < 0.05$) in

irradiated plasma-injected mice than in irradiated controls. The total blood leukocyte and blood neutrophil concentration did not differ significantly between injected and noninjected irradiated mice.

To determine whether there were differences in iron uptake in parts of the body other than the spleen after plasma injection, the following experiment was performed. Mice were given iron-59 intravenously 6 hr before death 10 days after irradiation; after death they were dissected and iron uptake into various parts was determined. In a pilot experiment, it was demonstrated that 80% of the iron in skinned, intact legs was recoverable in dissected bone. It was assumed that iron uptake in legs, head, thorax, and pelvis was representative

TABLE VII

*Effect of Plasma Injection Upon the Number of Nucleated Cells in Marrow, Volume of Packed Red Cells (VPRC) and Blood Leukocyte Concentration in Irradiated Mice**

Days of plasma injection before irradiation	Colonies per spleen	Nucleated marrow cells (million per humerus)	VPRC	Blood leukocyte	Blood neutrophils
			%	*per mm³*	*per mm³*
0	2.8 ± 0.8‡	1.51 ± 0.53‡	39.6 ± 0.8‡	460 ± 50‡	260 ± 52‡
4	29.7 ± 2.5	1.81 ± 0.64	43.4 ± 0.9	580 ± 62	170 ± 30
8	>30.0§	—	43.9 ± 0.8	510 ± 49	250 ± 48

* Measurements made 10 days after 700 R, normal dog plasma, 0.5 ml, injected intraperitoneally.

‡ Mean ± standard error for group of 10 mice.

§ All spleens in group with confluent colonies.

primarily of bone marrow iron. The only statistically significant difference observed between plasma injected and noninjected mice (20 per group) was iron uptake into spleen (4.2 vs. 0.6%). The total uptake by legs (6.0 vs. 8.9%), head (11.9 vs. 12.7%), thorax (12.3 vs. 14.1%), and pelvis (19.3 vs. 15.0%) was not significantly different in plasma-injected mice when compared to saline injected mice.

The Effect of Mouse, Rat, and Human Plasma.—Plasma from isogeneic mice and plasma from the parent strains (C57BL and DBA) induced no significant change in splenic hematopoiesis when injected for 4 days before irradiation. However, plasma from Swiss-Webster mice induced a significant increase in postirradiation hematopoiesis when injected in the same dosage as the ineffective isogeneic plasma (Table VIII).

Human plasma and rat plasma were quite effective as stimulants to postirradiation splenic hematopoiesis (Table VIII). Plasma from five different human subjects was tested and, while plasma from all subjects was quite effective, the magnitude of the effect was quite different from one subject to

another, a phenomenon similar to that noted with different dog plasmas (Table V). Plasma from these same five subjects was collected and tested again 2 months after the original test. Again, there was significant variation between the plasmas but the relative effectiveness of plasma from individual donors was

TABLE VIII

The Effect of Mouse, Rat, and Human Plasmas and Human Plasma Fractions Upon Splenic Hematopoiesis in Irradiated Mice*

Type of plasma	Volume injected per day	Mice per group	Splenic hematopoiesis		
			Spleen weight	Iron	Colonies
			Ratio of experimental group to control		
	ml				
Mouse					
Isogeneic	0.25	20	1.0	1.6	0.9
C57 BL (maternal)	0.25	20	0.9	1.1	0.6
DBA (paternal)	0.25	10	0.9	0.7	0.5
Swiss-Webster	0.25	10	1.6‡	2.9	3.4
Rat (Holtzman)	0.5	10	1.4	7.0	5.5
Human§	0.5	50	4.2	47.2	>30
Human plasma fractions‖					
Albumin	0.1‖	10	1.1	1.6	1.8
Gamma globulin	0.5‖	10	1.1	1.0	1.4
Fibrinogen	0.5‖	10	1.4	7.2	6.8
Dog serum	0.5	10	1.8	5.2	7.3

* Four consecutive daily injections intraperitoneally preceding 700–750 R in the various studies which were combined to make up the table.

‡ Underlined numbers differ by P = <0.05 by t test from paired, irradiated control.

§ Plasma from 5 different human subjects was tested, see text.

‖ See Materials and Methods for source and concentration of plasma fraction.

not reproducible. For instance, the donor whose plasma was most effective in the first test had the least effective plasma in the second test.

Crude fractions of human albumin and gamma globulin, injected in a dose exceeding that in the volume of human plasma injected (see Materials and Methods) did not induce a significant increase in splenic hematopoiesis (Table VIII). A crude fraction of fibrinogen was effective but fibrinogen cannot be solely responsible for the effect of foreign plasma. Dog serum, containing no detectable fibrinogen when tested by the addition of excessive amounts of thrombin, proved effective (Table VIII).

The Effect of Endotoxin and Other Foreign Materials upon Spleen Colony Formation.—The possibility that the effect of plasma was due to contamination by endotoxin was investigated. Infusion of 300 ml of the pooled normal dog

TABLE IX

*The Effect of Various Foreign Materials upon Splenic Hematopoiesis in Irradiated Mice**

Foreign materials	Mice per group	Splenic hematopoiesis		
		Spleen weight	Iron	Colonies
		Ratio of experimental group to control		
Endotoxin‡				
0.0001, 0.001, 0.1 and 1.0 μg/day × 4 days	50	1.2	1.1	0.7
10 μg/day × 4 days	20	1.5§	8.6	5.7
25 μg/day × 4 days	10	2.1	14.3	25.2
50 μg/day × 4 days	10	2.0	14.3	24.2
25 μg on day −1	10	2.1	19.5	23.7
25 μg on day −2	10	1.1	8.3	6.8
25 μg on day −3	10	1.7	12.9	15.7
25 μg on day −4	10	1.4	7.6	14.3
Misc. substances (× 4 days)‡				
Sheep red cells, 0.25 ml/day	10	1.2	2.4	2.1
Mumps vaccine, 0.1 ml/day	10	1.5	4.7	8.2
Influenza vaccine, 0.1 ml/day	10	1.4	3.7	6.0
Phytohemagglutinin, 0.5 ml/day	10	1.4	6.2	8.0
Cortisol‡				
10 mg, day −4	10	0.9	1.3	1.2
10 mg/day × 4 days	10	0.9	0.8	1.4
Dog plasma × 4	10	1.6	16.6	18.4
Dog plasma × 4 + 10 μg cortisol day −4	10	1.4	13.7	12.4
Dog plasma × 4 + 10 μg cortisol/day × 4	10	1.4	11.8	8.6‖

* Mice studied 10 days after irradiation exposure of from 650 to 750 R in various experiments combined herein.

‡ See Materials and Methods for details of preparation and concentration of injected substances; all injections were given before irradiation.

§ Underlined numbers differ from paired, uninjected, irradiated control by $P = <0.05$ by t test.

‖ Differs from plasma injected group *not* given cortisol by $P = <0.05$. Other cortisol + plasma group results do not differ significantly from groups treated with plasma alone.

plasma used herein in normal dogs induced neither neutrophilia or lymphopenia in the recipient. As little as 0.1 μg of the endotoxin used herein will induce lymphopenia in the dog (6). Thus, the maximal concentration of endotoxin of equivalent potency in this dog plasma should not exceed 10^{-3} μg/ml. Neither

that dose (10^{-3} μg) nor 10^{-2}, 10^{-1}, and 1 μg had any effect on spleen colonies (Table IX). However, still larger doses of endotoxin did induce a significant increase in colonies with maximal effect being achieved by 25 μg per day. No further increment was induced by doubling that dose (Table IX).

Smith et al. (13) reported that a single dose of endotoxin given 24 hr before irradiation induced a larger increase in spleen colonies than did repetitive doses preceding X-ray. We therefore compared single doses of endotoxin on day −4, −3, −2, and −1 with repetitive doses on each of those days. Our results were similar to those of Smith. Although a single dose given on any of the 4 days preceding irradiation had a significant effect, a single dose on day −1 proved most effective and indeed was as effective as were 4 repetitive doses (Table IX). This is quite different from the effect of dog plasma.

Phytohemagglutinin, sheep red cells, mumps vaccine, and influenza vaccine were all effective stimuli for increasing spleen colony formation (Table IX).

Cortisol, given as a single dose or for 4 consecutive days before irradiation, did not effect splenic hematopoiesis, but had an equivocal effect of reducing splenic response to dog plasma when given before or with plasma injections (Table IX).

DISCUSSION

The number of spleen colonies developing in mice 10 days after irradiation was increased by preirradiation injection of dog plasma and serum, human plasma and a crude fraction of human fibrinogen, rat plasma, Swiss-Webster mouse plasma, sheep red cells, phytohemagglutinin, mumps and influenza vaccine, and endotoxin. No effect was demonstrated after injection of isogeneic mouse plasma, parent strain (C57BL and DBA) mouse plasma, cortisol, and crude fractions of human albumin and gamma globulin. In certain experiments, the number of colonies was increased to more than 30 times that observed in uninjected irradiated controls.

This profound effect becomes of considerable interest when the nature of spleen colonies is considered. In all probability, each colony is representative of the progeny (erythroblasts, neutrophils, and megakaryocytes) of a single, pluripotential, hematopoietic, stem cell (14–16). Thus, the elucidation of details and mechanisms of the changes induced in spleen colonies may lead to a better understanding of this stem cell compartment. Spleen weight and radioactive iron and iododeoxyuridine (IUdR) uptake by the spleen were increased in proportion to the increase induced in colony number. This suggests that the induced increase in colony number was unaccompanied by a change in individual colony size or cellular composition. Morphological examination of selected spleens was compatible with this conclusion.

In plethoric, plasma-injected mice there was an increase in number of colonies comparable to that observed in nonplethoric, plasma-injected mice. In ple-

thoric mice, with or without plasma injection, spleen colonies were tiny and composed of granulocytes primarily. Splenic iron uptake in injected or non-injected (1) plethoric mice was indistinguishable from that of an irradiated spleen without colonies, confirming the absence of significant erythropoiesis in the spleens of such mice. However, the plasma-induced increase in colonies in plethoric mice was not detected by measuring spleen weight and IUdR uptake. This suggests that these measures reflect erythropoiesis primarily, that erythroid cells predominate in colonies (15, 16), and that changes in granulocyte proportions in colonies from nonplethoric animals are difficult to detect by these means.

Total, postirradiation, effective erythropoiesis appeared to be increased by plasma injection for the VPRC was increased in such animals when compared to controls. Since no detectable change was induced by plasma in marrow hematopoiesis, the increased erythropoiesis was apparently limited to the spleen. Brecher et al. (17) noted that the spleen may be the primary source of erythrocyte production at certain stages of postirradiation recovery.

The use of iron uptake, IUdR uptake, and spleen weight extends the measurable range of effect of an injected substance upon postirradiation hematopoiesis beyond that obtainable by counting spleen colonies alone. If more than 30 colonies are present, they tend to be confluent and therefore uncountable. Spleens with confluent colonies weigh about 45 mg in the strain of mouse used herein, but spleen weight, iron uptake, and IUdR uptake continued to increase in parallel until a spleen weight of approximately 60 mg was reached. Popp et al. (18) noted that spleen weight continued to increase at the same rate for some time after a level of confluent colonies was reached in the transplant system.

Elucidation of the substance(s) in plasma leading to its effect upon spleen colonies and the mechanism by which this effect is mediated will require further study but certain observations warrant discussion in this context.

First, the question must be raised whether the effect of plasma may be representative of the presence of physiologic growth-stimulating substances in plasma. Other investigators (2, 3) have reported changes induced in spleen colonies by administration of such materials as extracts of foreign kidneys and have suggested that the induced change is representative of substances controlling growth of granulocytes. Unfortunately, this attractive hypothesis must probably be rejected as an explanation for the effect of plasma. Isogeneic and allogeneic plasma from closely related mice was ineffective, whereas allogeneic plasma from Swiss-Webster mice was quite effective. There is no reason to believe that growth-regulating factors should be present in the plasma of one strain of mice, and in dog, human, and rat plasma, but be missing in the plasma of three other strains of mice.

The second general mechanism of action which can be suggested is that

foreign plasma is inducing an increase in spleen colonies through its antigenic effect. All of the effective materials studied would be expected to be antigenic when injected into mice. However, certain inactive materials such as human albumin and gamma globulin should also be antigenic. Furthermore, the dose-response relationships observed between injection of dog plasma and increase in colonies were not characteristic of response to an antigenic stimulant. A single dose of antigen, given 4 days before testing is as effective a stimulus to antibody response as are four consecutive injections (19). Such single dose schedules were ineffective in our system. Three single injections, given at 1 wk intervals, is a standard method of inducing an antibody response. Such a schedule was ineffective in inducing an increase in spleen colonies. Finally, secondary antibody responses are less affected by irradiation than primary responses. Yet, prior exposure to plasma and irradiation did not influence the magnitude of the effect of plasma when given before a second irradiation (Table VI).

The presence of relatively undefined "heterophile" antibodies in the injected plasma might, however, play some role in the effect of plasma upon splenic hematopoiesis.

The third possibility that warrants discussion is that foreign plasma induces a change in spleen colonies through its ability to induce an inflammatory response following injection (20). Cortisol markedly reduces inflammatory reactions (21) so that the slight reduction in the effect of plasma achieved by concurrent administration of cortisol is compatible with this hypothesis. This effect of cortisol is also compatible with an immunologic mechanism. However, the increased effectiveness of plasma when given intravenously as compared to intraperitoneally or subcutaneously is somewhat difficult to reconcile with an inflammatory effect.

The various substances which were shown to influence colonies need not act by the same mechanism. The maximal effect of endotoxin was obtained by a single injection 1 day before irradiation, while the effect of plasma was maximal when given as multiple sequential doses, suggesting a different mechanism of action for these substances.

In studies reported to date (13, 22–24), an induced increase in endogenous spleen colonies has usually, although not invariably (25), been correlated with an increase in radiation survival. If all of the substances which increase colonies also increase survival after radiation, the uniqueness of the effect of "radioprotective chemicals" (13, 22, 24, 26–28) must be questioned.

However, an increase in spleen colonies need not represent, necessarily, an increase in the "total body colony-forming cell pool." The total size of this diffusely located compartment has not been assessed. Transplantable hematopoietic colony-forming cells have been demonstrated in bone marrow (29), spleen (29), fetal liver (29), peritoneal washings (30), and blood (30). They have not been demonstrated in adult liver (29), in normal fetal or adult thy-

mus (31), lymph nodes (30), or thoracic duct drainage (30). In irradiated mice in which a limb (32) or the tail (33) is shielded, there is a rapid migration of colony-forming cells from the shielded area. Whether the majority of colony-forming cells in normal animals or whole body irradiated animals are migrating from one to another site is unknown. Thus, the increase in spleen colonies observed in response to plasma could represent an increase in the number derived from the blood, which lodge and grow in the spleen, or be due to an increase in the number of cells, eventually forming colonies, which were in the spleen at the time of irradiation.

In view of the ease with which hematopoietic spleen colony formation has been influenced by the injections of various materials reported herein as well as by those reported elsewhere (13, 34–37), it is apparent that rigorous controls must be studied before any conclusions can be reached about the presence of physiological regulating substances in the injected material. The ineffectiveness of normal isogeneic mouse plasma is encouraging in this regard, for it provides a proper control for the search for such factors in the plasma of perturbed mice. Furthermore, since certain plasma fractions were inactive, it may prove possible to remove activity from foreign plasma and still search for physiological growth-stimulating factors therein.

SUMMARY

Normal dog plasma and serum, human, rat, and Swiss-Webster mouse plasma, phytohemagglutinin, sheep red cells, mumps and influenza vaccine, fibrinogen, and endotoxin injected before irradiation led to an increased number of endogenously derived spleen colonies in irradiated mice. Spleen weight and uptake of radioactive iron and iododeoxyuridine into such spleens were also increased. The relationship between these parameters of splenic hematopoiesis was unchanged by plasma injection suggesting that, while the number of colonies was increased, the composition of individual colonies was unchanged. This conclusion was supported by studies on plethoric mice in which splenic erythropoiesis is abolished. Increased splenic hematopoiesis was accompanied by an increase in the volume of packed red blood cells 10 days after irradiation.

The total volume of plasma injected, the number of days of plasma injection preceding irradiation, and the route of administration were all important variables influencing the effect of plasma injections.

Crude fractions of human albumin and gamma globulin, cortisol, C57BL (maternal) and DBA (paternal) mouse plasma, and isogeneic plasma were without effect. The ineffectiveness of isogeneic and closely related allogeneic plasma rendered unlikely the hypothesis that this effect represented the presence of homeostatic hematopoietic regulating factors in plasma.

The increased hematopoiesis induced with plasma appeared to be limited to the spleen, for increased bone marrow hematopoiesis was not detected.

Certain observations suggested that the effect of plasma may not be due to

an antigenic or an inflammatory effect. From current observations, it was unclear whether the increased colonies induced by plasma were representative of expansion of the colony-forming cell pool or of increased efficiency of growth of the fraction surviving irradiation.

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