Spleen-Colony Formation in Anemic Mice of Genotype WW^v

Abstract. *The hemopoietic cells from anemic mice of genotype* WW^v *are less able by 200-fold to take part in colony formation in the spleen than cells from the normal littermates of genotype* ww. *The genetic defect shows itself in the colony-forming cells, since cells from normal littermate mice form colonies in the spleens of unirradiated mice of genotype* WW^v. *Use of animals of genotype* WW^v *as recipients improves the spleen-colony method by removing bias resulting from the death of irradiated recipients.*

A macrocytic anemia occurring in mice of genotype WW^v has been studied extensively (*1, 2*). A remarkable feature of the anemia in these animals is that it can be cured permanently by the intravenous infusion of hemopoietic tissue from isologous fetal liver from animals of genotype *ww* (*3*). The marrow spaces of the recipient anemic mice become populated by the descendants of the infused cells, and normal numbers of peripheral erythrocytes result from the proliferation and differentiation of these cells. Thus, isologous marrow grafts can grow and function in mice of genotype WW^v in much the same way as in heavily irradiated hosts.

Mice of genotype WW^v are very much more susceptible to the short-term lethal effects of total-body x-irradiation than are mice of the normal *ww* genotype. Bernstein (*4*) found the LD 50/30 (lethal to 50 percent in 30 days) for the anemic mice to be in the range 250 to 350 roentgens, in contrast to values near 700 to 750 roentgens for their normal littermates. However, WW^v anemic mice successfully implanted with isologous normal *ww* blood-forming tissues show LD 50/30 values approaching those of normal *ww* individuals (*5*), indicating that mice of genotype WW^v might be deficient in those cells which promote survival in irradiated *ww* animals.

We have suggested (*6, 7*) that the cells responsible for regenerating the hemopoietic tissues and hence for survival of irradiated mice may be recognized by their capacity to form macroscopic colonies in the spleens of mice irradiated with 900 roentgens of x-rays. These cells have been termed "colony-forming" cells, since, at present, they can be detected only by this property. Three types of evidence have indicated that the colony-forming cells play a part in the survival of animals exposed to total-body radiation. (i) The cellular descendants of colony-forming cells include the erythrocytic, granulocytic, and megacaryocytic precursors whose functions are necessary for life (*8*). (ii) The kinetics of repair of colony-forming capacity in the spleens of mice exposed to sublethal doses of total-body radiation appears to correlate well with the kinetics of repair of radiation injury as measured by changes in the LD 50/30 (*6*). (iii) The LD 100/30 (*9*) is approximately equal to the dose required to eliminate colony formation by "endogenous" colony-forming cells (*10*) present in the spleens of animals exposed to total-body radiation. The hypothesis that colony-forming cells are important in the regeneration of hemopoietic tissue, taken together with the work of Bernstein (*3–5*), led to the predictions that anemic mice of genotype WW^v would be deficient in colony-forming cells, and that transplants of normal, isologous hemopoietic cells might be able to colonize the spleens of unirradiated anemic mice. Our results indicate that both these predictions were correct.

Severely anemic mice of genotype WW^v, along with their hematologically normal littermates of genotype *ww*, were obtained from the Jackson Laboratory, Bar Harbor, Maine. These mice were hybrids obtained by crossing animals of the genotype WB-*Ww* with mice of genotype C57BL/6-W^vw. Thus, littermates may be considered to differ from each other genetically only at the W locus. In addition, C57BL/6 mice obtained from the annex colony of the Ontario Cancer Institute were used.

Table 1. Assay for colony formation by cells of normal and genetically anemic mice.

Genotype	Cells recovered per spleen or two femora	Nucleated cells injected	Spleen colonies ± std. error	Colonies per 10^5 cells
		Femoral marrow		
ww	4.4×10^7	1.0×10^5	12.2 ± 2.3	12.2
WW^v	3.0×10^7	8.5×10^6	0.4 ± 0.1*	0.005
		Spleen		
ww	1.7×10^8	5.0×10^5	4.1 ± 0.9	0.82
WW^v	1.7×10^8	3.0×10^7	1.1 ± 0.3*	0.004

* Colonies were small in size and difficult to count.

Table 2. Colony formation by *ww* marrow cells in unirradiated WW^v hosts and irradiated C57BL/6 (IC hosts).

Host	Surviving/ initial	Cells injected ($\times 10^4$)	Colony counts per spleen*
		Experiment 1	
WW^v	5/5	2	2, 0, 3, 4, 2
WW^v	5/5	4	6, 5, 4, 4, 8
WW^v	5/5	6	9, 8†, 4, 5
WW^v	4/4	8	6, 8, 7, 10
IC	3/42	10	10, 10, 13
		Experiment 2	
WW^v	5/5	5	5, 5, 4, 3, 1
WW^v	5/5	10	7, 4, 7, 8, 3
WW^v	4/5	15	12, 14, 9, 10
WW^v	4/5	20	7, 17, 13, 10
IC	5/48	8	9, 15, 5, 1, 6

* The standard errors of the mean of the colony counts in experiment 2 are 0.8, 1.0, 1.1, 2.1, and 2.3 respectively. Those of experiment 1 are plotted in Fig. 1. † Not countable.

The spleen-colony method of assaying for the proliferative capacity of cells derived from hemopoietic tissue has been described (*11, 12*). In the first experiment, we tested for the capacity of cells derived from mice of genotype WW^v to form colonies in irradiated C57BL/6 recipients. Accordingly, cell suspensions were prepared from the marrows (*11*) and spleens (*13*) of groups of nine to ten donors of genotype WW^v and *ww*, and each suspension was injected intravenously into a group of heavily irradiated (900 to 1000 rad) C57BL/6 mice. After 9 to 11 days, the survivors were killed, their spleens were fixed in Bouin's solution, and visible colonies were counted. From Table 1, it is evident that the yield of nucleated cells obtained from marrow and spleen of normal and anemic mice was very similar, and that cells obtained from mice of genotype *ww* had normal colony-forming capacity, since the number of colonies observed per 10^5 cells injected was similar to that observed in other strains (*11, 13*). In contrast, cell suspensions from mice of genotype WW^v were very deficient in colony-forming capacity since only a few very small colonies were observed even when large numbers of nucleated cells were injected. Of these, it is possible that a proportion were the

result of survival of endogenous colony-forming cells derived from the hemopoietic tissues of the heavily irradiated recipient mice, though the average number of surviving endogenous colonies after a total-body radiation dose of 900 rad is usually considerably less than one colony per spleen (*6, 10*). In any event, it may be concluded that the colony-forming capacity of cells from anemic mice is less by at least 200-fold than that of cells from their normal littermates.

The results shown in Table 1, coupled with the demonstration by Bernstein and Russell (*3*) that normal cells from mice of genotype *ww* can be transplanted into unirradiated hosts of genotype WW^v, suggested that colony-forming cells from normal *ww* mice might give rise to colonies in the spleens of unirradiated, anemic mice. Therefore, known numbers of femoral marrow cells obtained from ten mice of *ww* genotype were transplanted into groups of unirradiated mice of genotype WW^v; after 10 days the recipient animals were killed and their spleens were fixed in Bouin's solution. Figure 1 shows a spleen of an unirradiated WW^v mouse 10 days after intravenous injection of 2×10^5 marrow cells from normal *ww* mice. It is apparent that colonies analogous to those observed in irradiated hosts were formed in the spleen of the unirradiated WW^v recipient. Thus WW^v mice might prove valuable as recipients in the techniques of assaying spleen-colony formation, since all of these unirradiated animals might be expected to live for the 10 days necessary for macroscopic colonies to be formed, whereas many irradiated recipient animals would die during this period (Table 2). However, their usefulness as recipients would depend on the demonstration of a linear relationship between the number of nucleated cells injected and the mean number of colonies per spleen observed. The results of two experiments in which this linear relationship is demonstrated are presented in Table 2.

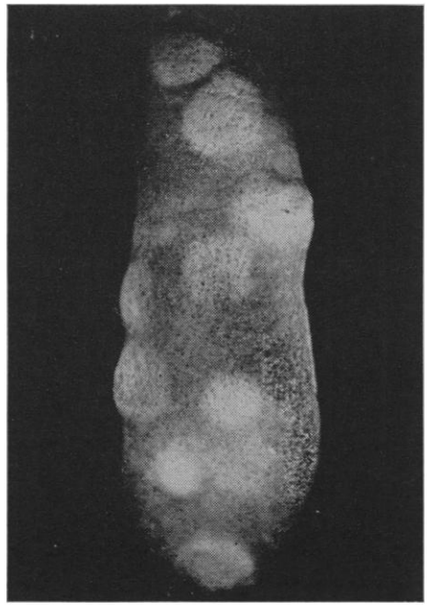

Fig. 1. Colonies in the spleen of an unirradiated mouse of genotype WW^v, which had been injected 10 days previously with 2×10^5 marrow cells of normal *ww* genotype. The spleen was fixed in Bouin's solution.

Figure 2 shows that a linear relationship exists between the number of nucleated cells injected into WW^v mice and the number of colonies observed, similar to that found when irradiated mice were used as recipients (*11, 12*). Further, it may be seen that the mean number of colonies observed per 10^5 cells injected when unirradiated WW^v mice were used as recipients was not significantly different from the mean number when irradiated C57BL/6 animals were used (Table 2).

The results indicated that mice of the genotype WW^v are suitable as recipients for the measurement of colony formation by hemopoietic cells from littermate animals with genotype *ww*. Further, these nonirradiated recipients have the advantage over irradiated normal recipients in that the results obtained are not biased because of loss of animals from death by irradiation during the period of the assay, and fewer animals per group will yield results of equivalent precision to those obtained with irradiated hosts. It is anticipated that this improvement in the spleen-colony assay technique will broaden greatly the applications which may be made of this method in the investigation of hemopoietic cell function (*14*). Our results support the view that colony-forming cells play an important role in the regeneration of hemopoietic tissue after total-body radiation. Anemic WW^v mice fail to recover from modest doses of total-body radiation (*4, 5*), and cells from the hemopoietic tissues of these mice fail to form spleen colonies when injected into irradiated, genetically normal, animals. The genetically anemic mice are capable of supporting colony formation by cells derived from mice

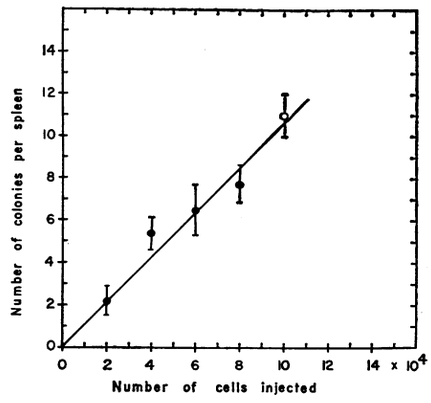

Fig. 2. Relationship between the mean number of colonies per spleen and the number of normal (genotype *ww*) marrow cells injected. Closed circles: unirradiated WW^v recipients. Open circle: irradiated C57BL/6 recipients. Vertical lines are standard errors. Data from experiment 1, Table 2.

of normal genotype. Thus, the hemopoietic tissues of genetically anemic mice either are deficient in cells with colony-forming ability, or they contain cells which require a stimulus for colony formation which is not provided under the conditions of our experiments.

Russell and co-workers (*15*) have demonstrated that erythropoiesis is defective in irradiated WW^v mice. Since normal spleen colonies contain large numbers of erythrocyte precursors (*8*), a deficiency in erythrocytic differentiation could prevent normal colony development. Thus, it is possible that the reduced colony-forming ability of cells from WW^v hemopoietic tissue is a reflection of inadequate erythropoiesis. However, deficient WW^v hemopoietic tissue is still able to provide the continuing supply of cells necessary for maintenance of a constant, though reduced, red cell population and a nearly normal marrow cellularity (*2*). The anemic mice are also able to respond well to the erythropoietic stimulus of hypoxia (*16*) and, to a much lesser extent, to the administration of exogenous erythropoietin (*17*). Our results support the suggestion (*18*) that the "stem cells" responding to erythropoietin (*19*) are not identical with the "stem cells" that give rise to spleen colonies.

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Immunologically Determined and Competent Cells Are Affected Differentially by Actinomycin D

Abstract. *A differential effect of actinomycin D on the capacity of mouse peritoneal fluid cells to form antibody after cell transfer has been found. Actinomycin treatment in vitro during transfer inhibited the delayed response toward a first antigen, administered before transfer, while the competence of cells to respond to a second antigen, given after transfer, was not affected.*

Cells specified in their capacity to produce antibody towards an antigen which they had encountered previously, may be called immunologically determined. They are contrasted with immunologically competent cells, which are able to respond to any new antigen they might encounter later on. In a system which allows both cellular states, determination and competence, to be measured within the same cell population, it is possible to do experiments designed to differentiate these two properties at their cellular chemical basis. In the work reported here, actinomycin has been used because of its known interaction with DNA, leading to an inhibition of DNA-directed RNA synthesis (*1*). It will be shown that antibody formation resulting from cellular determination is abolished after an incubation of cells with actinomycin, while competence is retained.

Mouse peritoneal fluid cells, harvested, washed, and transferred to lethally irradiated isogenic recipient mice, can produce antibodies of two different specificities: (i) against an antigen that has been given to the cell-donor mice before transfer, and (ii) against a different antigen that is administered to the recipient mice immediately after transfer. The first antigen may be given to the donor mice as early as 3 months before transfer. Antibody synthesis commences in the recipient mice after a lag period of 4 days after transfer, although the first antigen has not been administered again. Thus the retention by these cells of the capacity to begin antibody production against the antigen encountered earlier, not as in a secondary response, but without further antigenic stimulation, demonstrates immunological determination. The capacity of the same cell population to respond to the second antigen, given only after transfer, reflects the presence of immunological competence. During transfer in vitro the cell populations were treated with actinomycin, which thus acts on the cellular state of determination with respect to the first antigen, and on competence with respect to the second. Since peritoneal fluid cells are a heterologous population, it is not known whether determination and competence are properties of the same or of different cells. Facts and arguments leading to the concept of immunological determination will be summarized.

All mice were C3H/HeICR females 8 to 10 weeks old at the beginning of experiments. The two serologically unrelated *Escherichia coli* phage species, $T6r^+$ and T7, were used interchangeably as the first and the second antigen, respectively. The immune peritoneal fluid cells were produced in female C3H mice which received the following sequence of treatments: (i) a dose of 800 rad whole body x-radiation (40.5 rad/min, 190 kv, 20 ma, filtered by 0.5 mm Cu, 1 mm Al, target distance 50 cm); (ii) within 4 hours after the irradiation an intravenous injection of 40 to 60×10^6 spleen cells from previously immunized isogenic mice, prepared 4 days after these mice had received the second of two intraperitoneal injections of 10^{10} plaque-forming units of bacteriophage (the first antigen) 3 to 5 weeks apart; the first injection had been given in complete Freund's adjuvant (Difco); (iii) an intraperitoneal injection of 10^{10} plaque-forming units of phage (first antigen) in 0.2 ml complete Freund's adjuvant. Ascitic fluid developed within 10 weeks after a single injection of phage (T7) in adjuvant, or within 2 to 4 weeks after a second injection (T6), given 4 weeks after the first. The cells were sedimented from the ascitic fluid, resuspended with the help of 0.025 percent Trypsin (Difco), and washed three times by a sucrose-layer technique. Thereby the amount of antibody carried over into the recipients, extra- or intracellularly, became too small to interfere with the measurement of antibody synthesized by the cells after transfer (Fig. 1 and ref. *7*). The incubation with actinomycin D was between the second and third washings, in a water bath at 37°C for 40 min. It was terminated by chilling the tubes with cells in ice water (*2*). The cell concentration during the incubation was 23×10^6 cells per milliliter. Within each experiment, cells incubated with or without actinomycin were portions of the same preparation of peritoneal fluid cells. Cell suspension medium was Hanks solution containing 2 percent polyvinylpyrrolidone (PVP, Cal-BioChem.) For the 37°C incubations, the bicarbonate-CO_2 buffer concentration was increased fivefold. The bottom layer in sucrose-centrifugation was a mixture of 2 volumes of Hanks-PVP and 1 volume of 0.6 molar sucrose in water.

The mice injected with immune peritoneal fluid cells received 850 rad, and then were injected intravenously with 10.5 to 42×10^6 peritoneal fluid cells per mouse, to which 7 to 10×10^6 un-