Decrease in Numbers of Mouse Spleen Nodules with Time Post-Irradiation.* (31679)

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Spleen nodules which derive from isologous marrow cells transplanted to irradiated mice have been shown to be clonal in nature, *i.e.*, to arise from single transplanted cells(1). One would expect constancy in the actual number of such colonies during the initial period of their growth, and no decrease during this period in the numbers counted by gross observation of spleens removed on successive days. In agreement with such expectations are: a) the results of McCulloch(2) who reports an increase between the 7th and 9th days post-irradiation and constancy on days 9 through 13; b) the results of Lewis and Trobaugh(3) who found constancy in numbers on days 5 through 11; and c) the author's results in a marrow-transplant experiment reported here.

However, significant decreases in counts have been observed in experiments recently performed in this laboratory, with mice in which the nodules either were a) "endogenous," *i.e.*, induced by simple whole-body irradiation, or b) induced by a tail-shielding treatment. Since these decreases take place before the 8th day, the earliest commonly used for spleen assay, the phenomenon would ordinarily not be observed, and has not been reported in the literature, to the author's knowledge. The mechanism and significance of this effect are not understood.

Materials and methods. Two strains of mice were used, designated B and C in Table I. The B strain is the Brookhaven, Hale-Stoner, albino Swiss mouse which is maintained at this laboratory as an intact strain on a random breeding basis. The C strain is the hybrid C3H/Anf $\times$ 101 of inbred parent strains (Cumberland View Farms). The tail-shielding treatment, designated TS in Table I, consists of 2 exposures, each of the magnitude indicated, the first delivered to the body except for a shielded part of the tail, and the second, about 1½ hours later, to the tail only (4). The treatment designated BM consists of whole-body exposure of the magnitude indicated, followed within one hour by injection into each animal of 3×10^4 unirradiated, nucleated cells from the femurs of a single donor of the same sex and strain. Simple whole-body exposure is designated WB. Surviving mice were sacrificed according to prearranged schedule. Radiation mortality did not occur in most experiments, and did not exceed 4% in any.

Irradiations were made with 250 Kvp X rays of 1.2 mm Cu HVL at rates of about 110 R/min for the BM and WB treatments and 150 R/min for the TS treatment. The exposure values given closely approximate midline tissue values, since they are measured in a fully-scattering phantom, with the ionization chamber (Victoreen, 100 R) centered at the position of the tissue in question (trunk or tail).

Spleens were fixed in AAF (acetic acid, alcohol, formalin) except in Experiment 3 in reference. Diameters of round nodules were measured and counted, using low magnification together with a scale or reticle for reference. Diameters of round nodules were measured directly to the nearest 1/10 mm. Those of irregular shapes were assigned diameters, to the nearest 1/10 mm, of circles of areas equal to their calculated areas.

Differences between nodule counts of pairs of earlier and later groups within experiments were tested for significance by ranking the individual scores and applying the Mann-Whitney U-test. This test was chosen in preference to the t-test as being more applicable to groups with discrepant variances. The value of P given in Table I is an upper limit for the so-called "two-tailed" probability that an apparent difference (either increase or decrease) of such magnitude would occur on the basis of sampling, if the populations from which the groups were drawn did not actually

* Research supported by U. S. Atomic Energy Commission.

TABLE I. Comparisons of Numbers of Nodules on Earlier and Later Days Post-Irradiation.

Exp No.	Sex, age (wk),† strain‡	Exposure in R, and treatment‡	Day post-irradiation	Mice per group	Threshold diam., mm	Median nodule diam.,§ mm	Nodules per spleen, range	Variance ÷ mean $s^2/\bar{x}$	Mean nodules p. spleen $\bar{x}$	Groups compared, days	Ratio earlier $\bar{x}$ ÷ later $\bar{x}$	Probability limit‖ P
1	♂ 14 C	750 BM	4	6	0.1	.25	0- 8	5.0	2.0	5 & 8	1.0	N.S.
			5	10	"	.4	2-18	3.8	8.6			
			6	10	"	.55	5-30	5.2	10.2			
			7	10	"	.75	3-12	1.1	7.4			
			8	11	"	.9	5-12	.9	8.6			
2	♂ 16 C	750 TS	5	12	"	.25	0-30	11.7	8.8	5 & 8	3.8	.05
			6	12	"	.45	0-22	7.4	6.8			
			7	11	"	.75	0-10	2.2	4.3			
			8	13	"	.95	0- 7	1.5	2.3			
3	♂ 9 B	750 TS	6	15	"	.5	7-31	3.2	16.3	6 & 9	3.0	.002
			9	17	"	1.2	2-12	1.3	5.5	(TS)		
		750 WB	6	13	"	.7	0- 1	1.0	.1			
4	♂ 14 C	750 WB	5	24	"	.4	0-16	3.8	2.7	5 & 8	1.7	.02
			8	25	"	.5	0-14	6.4	1.6			
5	♂ 15 C	675 WB	5	20	0.2	.35	0-104	38.3	12.7	5 & 8	12.7	.002
			5*	19	"	.35	0-18	3.4	7.9	5* & 8	7.9	.002
			6	10	"	.4	0- 4	1.4	1.5	5* & 6	5.3	.002
			8	20	"	.25	0- 5	1.6	1.0			
6	♂ 14 C	675 WB	5	20	"	.3	0- 5	.9	1.7	5 & 8	1.3	.05
			8	20	"	.35	0-11	4.7	1.3	8 & 11	1.2	N.S.
			11	20	"	.3	0- 3	1.1	1.1			
7	♀ 14 C	600 WB	5	10	"	.35	5-86	13.4	48.0	5 & 8	10.9	.002
			6	9	"	.5	6-30	3.5	15.9	6 & 8	3.6	.002
			7	10	"	.5	2-26	6.9	8.6	5 & 6	3.0	.02
			8	12	"	.6	1- 8	1.3	4.4			
8	♀ 14 C	600 WB	5	11	"	.35	2-76	22.0	22.9	5 & 8	3.3	.02
			8	12	"	.65	1-29	10.2	6.9	8 & 11	.9	N.S.
			11	13	"	.8	1-17	2.5	7.7			

* Same as group directly above, except mouse with count of 104 (see *Discussion*).
† Ages given as 14 wk are half each 13 and 15 wk in each group.
‡ See *Materials and methods* for explanation of symbols.
§ To nearest 1/20 mm for all of diameter ≧ threshold.
‖ See *Materials and methods*. Symbol N.S. denotes $P > 0.05$.

differ. The "one-tailed" probability limit for the occurrence, in this case, of an apparent decrease, is one-half this value. As a measure of the variability within each group, the value of $s^2/\bar{x}$ has been tabulated, where s^2 is the so-called "unbiased estimate" of the variance, and $\bar{x}$ is the arithmetic mean or average. This ratio has a parametric value of unity in the case of a Poisson distribution of counts, such as would occur in a population in which nodule formation took place randomly, and with the same probability, in every mouse.

Results. Table I shows the results of 8 experiments: one of BM treatment, 2 of TS, and 5 of WB. The threshold diameter used in each experiment is given, as well as the median diameter and range of counts for each group. Earlier and later groups within each experiment are compared, both by the ratio of their mean counts and by the probability limit P as defined above.

Experiment 1, the marrow-transplant experiment, after an initial rise in nodule count, day 4 to 5, shows no significant change through day 8. The accompanying increase in median diameter is indicative of good nodular growth during this period.

Experiment 2, which has the tail-shielding treatment but is otherwise similar to Exp. 1, shows a marked and significant decrease in nodule count from day 5 to day 8, accompanied by an increase in median diameter.

Experiment 3, which is of treatment 750 TS as is Exp. 2 but with mice of different age and strain, includes a whole-body control group for day 6. This experiment shows a highly significant decrease in nodule numbers for the TS groups from day 6 to 9 accompanied by an increase in nodule size.

Experiment 4, with 750 R whole-body exposure, shows a small but significant decrease in nodule number from day 5 to 8, with little indication of growth in size. This experiment resembles Exp. 1 and 2 as regards strain, sex, age, and exposure, and accordingly serves as their whole-body control (see *Discussion*).

Experiments 5 and 6 are whole-body experiments with mice of the same strain, sex, and age as those used in Exp. 4, but with 75 R less exposure. The decrease between day 5 and day 8 is marked and highly significant in Exp. 5, and significant but less marked in Exp. 6. Exp. 5 shows a highly significant decrease from day 5 to day 6 (see *Discussion* for explanation of group 5*).

Experiments 7 and 8 are again whole-body experiments, using mice of the same age and strain as those in Exp. 4, 5 and 6, but of opposite sex and with less exposure. The decrease from day 5 to day 8 is marked and significant in both Exp. 7 and 8, highly significant in Exp. 7. Significant decreases are seen in Exp. 7 also for days 6 to 8 and 5 to 6. Exp. 8, like Exp. 6, shows no significant change from day 8 to day 11.

Discussion. In Experiment 1 the nodules counted can be considered to be the sum of those which originate from transplanted, unirradiated cells, and those arising endogenously from cells receiving 750 R. The latter appear to constitute a minor fraction of the total in the period 5 to 8 days as judged by the whole-body, but otherwise similar, Exp. 4. It would thus appear that the fraction of nodules arising from transplanted cells shows growth in size but little change in number during this period.

In Experiment 2, again using Exp. 4 as whole-body control, one may infer that most of the nodules seen on day 5 are derived from shielded tail cells, and that these subsequently undergo a decrease in numbers accompanied by growth in size.

In Experiment 3, using the 6-day control group, it appears that nearly all the nodules seen in the day 6, tail-shielded group are tail-derived rather than endogenous, and that these then undergo a decrease in numbers to day 9, accompanied by growth in size.

Experiment 5, because of the occurrence on day 5 of one unusually deviant, high spleen count, was given special statistical treatment, *i.e.*, it was analyzed without this count (group 5* in Table I) as well as with it. The degree of deviation of this count is such that when the mean and S.D. are calculated for the diminished group, 5*, the high value lies at 18.5 S.D.'s from the mean (note also the large change in $s^2/\bar{x}$ effected by exclusion of this count). Since statistically this indicates that the corresponding mouse belongs to a

different population from the rest of the group, and hence that the result may be biased, by its inclusion, in the direction of showing a false decrease, some comparisons are listed in Table I with this value excluded. The value of P for the comparison of 5* to 8 is 0.002 as is that for 5 to 8, and the P for the comparison of 5* to 6 is also 0.002 (as is that for 5 to 6, not shown in Table). Incidentally, no other reason for excluding this animal was evident. It did not differ significantly from the rest of the group as regards weight, loss of weight, spleen weight, appearance of viscera, or histology of its spleen (see ff.).

Serial sections were made of spleens from the 5 and 6 day groups of both Exp. 5 and Exp. 7. In general, the nodules showed many mitotic figures, few mature hemopoietic cells and little evidence of cell death as evidenced by pyknotic or fragmented nuclei. There was somewhat more evidence of cell death on day 6 in Exp. 5. The nodules in the sections of the high counting spleen of Exp. 5 did not appear to differ from those in other spleens of the same group. No "ghosts," *i.e.*, structures which might correspond to depopulated nodules, were evident in any of the spleens examined.

In connection with the 600 R whole-body Exp. 7 and 8 and in contrast to them, it is of interest to note the results of an experiment, not tabulated here, in which the same treatment was given mice which were also female but were of the B strain and 5 weeks younger. In this case a significant increase in mean nodule count was found from day 6 to 9. Preliminary data suggest that the strain, rather than age, difference is the more likely cause for this difference in effect.

The rapid decrease between day 5 and 6, seen in both Exp. 5 and Exp. 7, suggests a relatively synchronous disappearance of nodules which, together with possible straggle in phasing, could account for the discrepancy in the degrees of decrease found in Exp. 5 and 6.

It may be noted that the variability within groups as measured by the ratio $s^2/\bar{x}$ is, in general, greater on earlier days, and that in several instances it approximates unity, the Poisson value, on later days (see *Materials and methods*). This circumstance indicates a corresponding biological progression of the probability of nodule formation from a function which is highly variable within the group, to one which is relatively uniform. This trend, incidentally, tends to offset any statistical advantage that might be gained by assaying spleens on an earlier day with higher nodule counts, rather than on a later day.

In experiments in which a major fraction of the spleen surface is covered by nodules on the later days, it is quite possible to observe an artifactual decrease in numbers due to fusion. This situation was avoided in this work. The maximum fraction of the area covered by nodules on any spleen was about 10%, and fusion of nodules was relatively rare.

Conclusion. The experiments discussed above serve to illustrate the fact that decreases in numbers of nodules can occur following irradiation of mice either a) to the whole body, or b) with the tail shielded. The phenomenon is as yet unexplained, and there is little evidence to indicate the nature of concomitant events at the cellular level.

These findings might conceivably be related to the "abortive rise" in numbers of cells in bone marrow and peripheral blood, seen following whole-body irradiation of mammals(5). The abortive rise is thought to be an expression of cellular radiation damage. As such, it might be related to the decrease found with endogenous nodules, but not to that found with tail shielding, in which case the nodules arise principally from cells in the shielded tail.

A second effect, which suggests itself as being relevant because of its resemblance to the slow growth found in endogenous nodules, is the "small colony formation" reported by Sinclair(6) for cultures of heavily irradiated mammalian cells. This effect appears not to be related to the main phenomenon, however, since it is not accompanied by any decrease in colony counts.

The author wishes to thank S. L. Commerford, J. L. Bateman, and V. P. Bond for helpful discus-

sions and comment, and H. A. Johnson for help with morphology.

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Received September 19, 1966. P.S.E.B.M., 1967, v124.

Action of Isoproterenol on Heart Cells in Tissue Culture.* (31680)

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For many years it has been possible to grow dispersed heart cells in tissue culture, and rhythmic contraction of these cells has been noted(1). The rate of contraction of these heart cells has been measured by direct observation and electrical depolarization has been recorded by micropuncture techniques (2). The effects of cardioactive drugs, including epinephrine(3), quinidine(4), and digitalis(5), have been determined on these cells. Our studies were designed to assess the effects of drugs which stimulate or block specific adrenergic receptors in the heart(6). These experiments allow studies in an isolated system of cardiovascular tissue devoid of all neural connections.

Method. Beating hearts are removed from 9- to 11-day-old chick embryos. They are chopped into fragments and incubated for 20 minutes at 37°C in 0.125% trypsin in phosphate buffered saline containig glucose. The dissociating fluid is decanted and the settled cells resuspended in a modified Puck's medium. Dispersion of the cells is accomplished by gentle up and down pipetting. The larger tissue fragments are removed by filtering the cell suspension through a sterile mesh gauze. The cells are collected by centrifugation at 1500 RPM for four minutes and then resuspended in Puck's medium containing 20% horse serum and antibiotics. Approximately 5×10^5 cells in 2 ml of culture media are placed into 6 × 150 mm plastic plates. The cells are grown at 37°C in an incubator in a 5% carbon dioxide atmosphere. Cells attach to the bottom of the plates and rhythmic contraction starts in some cells within 24 hours. Medium changes are made every 2 days while the cells are developing. Many cells develop regular contractions at rates between 12 and 150 per minute. All studies are carried out at 3 to 6 days after plating the cells, although beating may continue to 9 or 10 days in some cell clusters.

The rate of contractions of cells was observed with a Zeiss Plankton microscope with inverted phase contrast optics. The observer records the contractions which occur in 2 one-minute periods. Increases and decreases in contraction rate were defined as changes greater than ± 3/minute. Cells were considered to have stopped when no spontaneous activity was noted for 2 minutes. All studies are carried out at 25° and all reagents are preincubated to that temperature. Drugs were added by medium change after they have been mixed with Puck's medium and all doses are reported as the base compound.

Results. The morphology of developing cells is illustrated in Fig. 1 and 2. No attempt to distinguish myocytes from other cell types was made except on the basis of their contractile properties. Myofibrillar structure was not noted in these cultures. As the cells aged, highly refractile inclusions are noted within their cytoplasm (Fig. 2).

Isoproterenol, a specific stimulator of beta adrenergic receptors(7) produces increases in the rate of contraction of cells (Table I). The threshold dose appears to be between 0.2 μg

* Supported in part by Grants HE 09058-03, AI-05629-04, and Training Grant 1 T1 HE 5709-01.