tant genes so far introduced into heterozygous diploid cells have been shown to be recessive to their wild-type alleles. In addition, the two alternative mating-type alleles *mt*− and *mt*+ have been tested for dominance. All the diploid cultures so far examined have been mating-type minus, indicating that *mt*− is dominant over *mt*+. To confirm this supposition a diploid heterozygote was selected from the mating *nic-7 mt*+ *ac-29*+ × *nic-7*+ *mt*− *ac-29* (*nic-7*, *mt*, and *ac-29* are very closely linked). From a cross of this heterozygote with the haploid wild-type strain, both *nic-7 mt*+ *ac-29*+ and *nic-7*+ *mt*− *ac-29* progeny were recovered. These results show that nonrecombinant derivatives of both parental chromosomes are present in the diploid, and support the original observation that *mt*− is dominant in cells heterozygous for mating type.

7) *Stability*. All diploid strains recovered so far have remained diploid in successive subcultures longer than 12 months. The fact that haploid cells have not been detected indicates that the cells of vegetative diploid strains do not undergo meiosis. However, mitotic recombination probably occurs, although rarely, since cells homozygous for one or more markers, but still heterozygous for other markers in the same linkage group, have been recovered.

The existence of diploid cultures of *C. reinhardi* provides a new tool for both formal and biochemical genetic investigations with this organism. Studies of mitotic recombination, and of the evaluation of units of genic activity defined by complementation analyses, are now possible with this organism.

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6 April 1967

Improved in vitro Survival of Normal, Functional Spleen Cells

Abstract. *An enrichment method for improving the viability of monolayers of normal mouse spleen cells is described. These cells are capable of saving lethally x-irradiated recipient mice from death, presumably by proliferating and differentiating into mature blood cells.*

Attempts to culture normal, functional hemopoietic or antibody-producing cells have been discouraging (*1*). We now report a method for development of such cultures. This method involves a process of selection or adaptation analogous to the enrichment culture procedures used in microbiology. It has also been successfully applied to cancer tissue in developing several clonal lines of functional cells (*2*).

Mouse spleen cells were inoculated into culture bottles and, after a few days, the small fraction of cells which survived was harvested. Because these cells had been passed in vitro once we refer to them as passage-1 cells or P_1 cells (*3*). These cells were then injected intravenously into mice which had been irradiated with a lethal dose of x-rays. The injected cells proliferated in the x-irradiated animal, repopulated the depleted hemopoietic organs, and thus saved the host from x-irradiation death (*4*). After 2 or 3 weeks, when the animals had partially recovered from irradiation, they were killed and their spleen cells were inoculated into culture bottles. Again, after 4 days in vitro these cells (P_2) were harvested and injected into lethally x-irradiated mice. This whole process was repeated to obtain P_3, P_4, and P_n spleen cells.

The choice of lethally x-irradiated recipients is a useful adjunct to this technique because it circumvents the host-versus-graft reaction. This process of serial in vitro and animal passage selectively enriches the cell populations which not only survive the conditions in vitro but also perform a life-saving function in the lethally x-irradiated recipients. Thus, briefly, two separate selective pressures are involved—one requiring viability in vitro, and the other requiring proliferation and differentiation in vivo into functional blood cells.

Parenchymal spleen cells of 8- to 10-week-old LAF_1 mice (Jackson Memorial Laboratory, Bar Harbor, Maine) were passed through a 40-mesh stainless steel screen and suspended in F_{10} medium supplemented with horse serum (15 percent) and fetal calf serum (2.5 percent) (*5*). Stromal cells remaining on the screen were discarded. A portion of the cell suspension was stained with crystal violet, and cells were counted in a hemocytometer. Approximately 10^8 nucleated cells were inoculated into 20 ml of medium in 150-ml tissue-culture bottles. The bottles were incubated at 37°C in a water-saturated atmosphere of 95 percent air and 5 percent CO_2. After 4 days, the small fraction of cells surviving was scraped from the bottles with a rubber policeman. Approximately 1.5×10^7

Table 1. Survival of lethally irradiated mice after intravenous injection of spleen cultures. All mice were irradiated with 770 to 850 roentgens and received 1×10^6 to 8×10^7 cells (most animals received 1 or 2×10^7 cells). Mice receiving the fewest cells had the highest mortality. All except one of the controls were dead by 21 days after irradiation. Most died from 10 to 14 days after x-irradiation.

Origin of hemopoietic cells	No. of injected mice		No. of uninjected controls	
	Total	Survivors	Total	Survivors
FSC*	5	5	8	0
P_1	19	12	7	0
P_2	14	13	12	0
P_3	13	12	6	0
P_4	7	5	8	1

* Freshly isolated spleen cells.

Table 2. Comparison of peripheral blood erythrocyte and leukocyte counts of normal mice with lethally x-irradiated controls and lethally x-irradiated experimental mice harboring various spleen cells passaged in vitro. RBC, red blood cells; WBC, white blood cells.

Origin of hemopoietic cells	Number of mice	10^6 RBC/mm³		WBC/mm³	
		Range	Mean	Range	Mean
P_0 (normal)	5	8.5–11	10.2	4000–14500	13000
P_2	5	6.6– 7.5	6.9	250– 4000	1200
P_3	5	6.0– 7.5	6.5	300– 3500	1000
P_4	4	6.0– 7.0	6.3	250– 900	450
X-irradiated controls	3	2 – 3.3	2.9		< 50

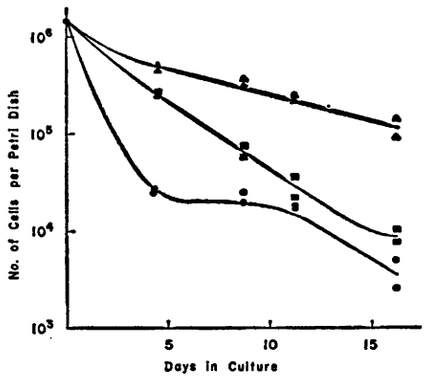

Fig. 1. Cells (1.5×10^6) derived from normal mouse spleen and the same number of cells derived from the spleen of x-irradiated recipients of spleen cells passaged in vitro were inoculated into individual petri dishes. At various times after the initiation of cultures, the attached surviving cells were harvested and counted in a hemocytometer. Cell viability was determined by impermeability to trypan blue after 5 minutes exposure to the dye. ●, Normal spleen; ■, passage-1 spleen; and ▲, passage-3 spleen.

of these cells were injected into the tail vein of each lethally x-irradiated mouse within 5 hours after irradiation. The mice received 770 to 850 roentgens of total body irradiation delivered under the 250 kv-peak, 1.0 mm of aluminum added filter, inherent filtration 0.54 mm copper and half-value layer of 1.23 mm copper. This dose of x-rays killed 99.5 percent of control mice by 21 days. The peak number of deaths occurred at 12 days. To test whether the alternate passage method improves the survival of the spleen cells in vitro, 1.5×10^6 normal spleen cells and the same number of cells from various spleens passaged in vitro (P_1, P_2, P_3, and so forth) were inoculated into 3 ml of medium in 50-mm plastic petri dishes. After various intervals, the attached surviving cells from duplicate culture dishes were counted. The difference in viability between normal, P_1, and P_3 cells can be seen in Fig. 1 where the number of surviving cells is plotted against time of survival in culture. This difference is also illustrated by a photomicrograph of representative fields of 1×10^7 normal cells as compared with the same number of P_2 cells, at 4 days in vitro (Fig. 2). The functional capacity of these cells was demonstrated by their ability to protect the mice from death by x-irradiation (Table 1). Counts of peripheral blood erythrocytes and leukocytes were made on representatives of each group of animals on the 14th day after x-irradiation (Table 2). It can be seen that the x-irradiated mice harboring various passaged cells in vitro have higher erythrocyte and leukocyte counts than x-irradiated controls.

Several investigators, in direct transfer experiments, have shown that mouse spleen cells, when transferred into lethally x-irradiated mice, give rise to erythrocytes and granulocytes of the donor type (*6*). If we assume that this is also true of our passaged spleen cells in vitro, it is reasonable to conclude that the higher erythrocyte and leukocyte counts noted in the recipients of these cells represents their proliferation and differentiation in vivo.

Siminovitch *et al.* (*7*) have shown that, when freshly isolated mouse spleen cells were directly injected into x-irradiated mice, the colony-forming ability and life-saving capacity of these passaged cells declined after several serial passages. This observation implies that the stem cells are capable of a limited number of divisions. If this is the case, it may not be possible to keep these cells as an established cell line, or to continue the selection procedure by serial culture and animal passage. However, the lack of proliferative ability in vivo has not been a problem in our experiments up to this time, as the cells have continued to proliferate and maintain hemopoietic capacity through five serial animal and culture passages. Whether or not a continuation of the present series of animal and culture passages can result in cells with proliferative capacity in vitro, as well as increased survival capacity, remains to be determined. It will also be necessary to ascertain the relative proportion of hemopoietic precursor cells as well as other cell types in these cultures with markedly improved survival in vitro.

The method described has resulted in cells which, under our culture conditions, do not express their capacity for proliferation and differentiation. The same cells express these potentials when they are put into the environ-

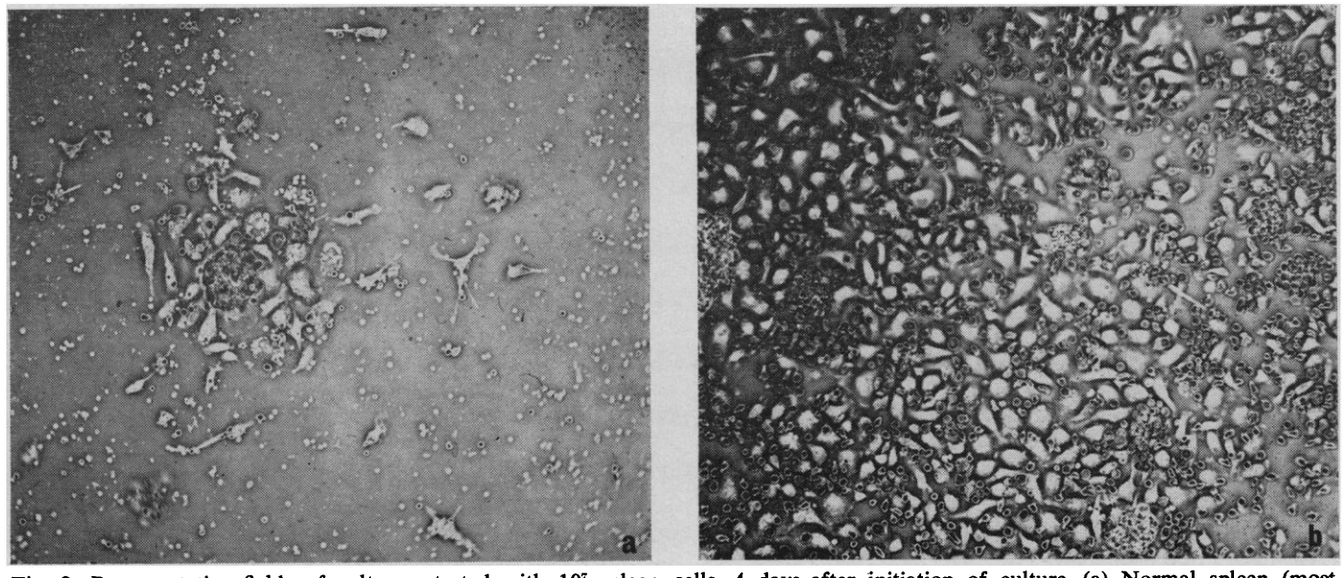

Fig. 2. Representative fields of cultures started with 10^7 spleen cells, 4 days after initiation of culture. (a) Normal spleen (most fields contained much cellular debris and no cells). (b) Passage-2 spleen cells.

ment of an x-irradiated mouse. The markedly improved survival in vitro of these cells offers an experimental system for the study of the factors which may be responsible for expression of these potentials in vivo or their repression in vitro.

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Function of the Dorsal Motor Nucleus of the Vagus

Abstract. *The dorsal motor nucleus of the vagus was destroyed in cats. After periods of survival ranging from 9 to 44 days the response of heart, bronchi, esophagus, and duodenum to stimulation of the distal end of the cut cervical vagus was within the normal range or slightly depressed. It is concluded that the dorsal motor nucleus is not a major source of visceromotor fibers.*

It is generally accepted that the dorsal motor nucleus (DMN) of the vagus is the source of visceromotor fibers to the heart, bronchi, and gastrointestinal tract. This concept is based on the observations of Marinesco (*1*) that retrograde chromatolysis reaches its peak in this nucleus 6 days after cervical vagotomy and that the neurons in the DMN do not have the cytologic characteristics of those of the hypoglossal and ambiguus nuclei, which are known to innervate somatic musculature. The relatively rapid onset of chromatolysis was, in Marinesco's opinion, a strong argument in favor of the DMN neurons' being motor rather than sensory, as had been proposed by previous workers. Cajal (*2*), who had regarded them as sensory neurons up to that time, reinvestigated the problem with the use of the Golgi technique and was able to follow their axons into the emerging vagal rootlets. Accordingly, he revised his opinions and concurred with Marinesco's view that neurons in the DMN were motoneurons that innervated visceral smooth muscle. This concept has remained almost unchallenged, with the exception of Szentagothai's (*3*) report in which he notes that after electrolytic lesions in the dorsal motor nucleus he was unable to find a single degenerating fiber in the intra-axial vagal rootlets. His observation was in conflict, however, with that of Cajal and with the occurrence of retrograde degeneration after vagotomy as described by Marinesco (*1*), a finding that has been confirmed by subsequent investigators, who also described a rostrocaudal organization of this nucleus according to the level at which vagotomy was performed (*4*). The possibility that the retrograde chromatolytic changes might be on a transynaptic basis is remote, because of the brief time course to maximum change, as pointed out by Marinesco (*1*), and because no terminal degeneration appears in the DMN after vagal rhizotomy (*5*).

Electrophysiological studies of the DMN that employ techniques for recording single units have only recently been reported. Urabe and Tsubokawa (*6*) found that these cells could not be fired antidromically from the cervical vagus, a finding that we [Kerr and Higgs (*6*)] noted several years ago at a time when we were unaware of their report. Calaresu and Pearce (*7*) have also raised the question whether at least some of the cardioinhibitory vagal fibers may have an origin other than the DMN, and before that several other authors had suggested that these fibers arose from the nucleus ambiguus.

In the experiments reported here, 20 adult cats were used. In the first stage of the experiments the floor of the fourth ventricle was exposed under nembutal anesthesia and the DMN was destroyed unilaterally by means of an incandescent filament 6 mm long and 0.08 mm (0.003 inch) in diameter. After periods of 9 to 44 days, the 15 surviving animals were reanesthetized, and responses of the heart, bronchi, and gastrointestinal tract to electrical stimulation of the cervical vagus, both ipsilateral and contralateral to the lesion, were monitored by means of Statham strain gauges attached to catheters in the aorta, esophagus, and duodenum and to the trachea. Respiratory exchange was controlled by a Harvard pump respirator that operated at frequencies of 8 to 12 per minute and with stroke volumes of 75 to 100 ml; bilateral thoracotomies were done in all animals.

Histological controls were made in every instance; serial frozen sections (20 μ thick) were made of the medulla in the transverse plane and were stained by the Luxol fast blue and Nauta techniques. In two experiments the DMN was widely destroyed, in a third all but about 40 neurons were destroyed and these showed variable degrees of damage. In all four experiments the DMN was uninjured, and in the remainder only partial destruction was obtained.

Figure 1 shows the result of stimulating the distal end of the cut cervical vagus (6 volts; pulse duration, 1 msec; frequency, 10 per second) 44 days after extensive destruction of the DMN. The cardioinhibitory vagal effect (tracing No. 1) is within the normal range of activity, the esophageal response (tracing No. 2) is present both as an "on" and an "off" response, and a marked duodenal response (tracing No. 3) is seen. The bronchiolar constrictor response (tracing No. 4) is unsatisfactory in this record, although normal bronchiolar constrictor responses have been recorded from similar experiments. The two bottom lines indicate onset and end of stimulation. The time line at mid-left corresponds to 10 seconds. On the left, blood pressure is given in millimeters of Hg, and esophageal and duodenal pressures, in centimeters of