Requirement of a Nonproliferating Class of Cells for Generation of Immune Responses in Cell Culture[1] (34667)

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(Introduced by E. A. McCulloch)
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Spleen cells from unimmunized mice exposed to xenogeneic erythrocytes will give rise to hemolysin-forming cells in cell culture (1, 2). Small numbers of CBA spleen cells fail to give rise to hemolysin-forming cells; however, they can produce hemolysin-forming cells when cultured in the presence of a large number of heavily irradiated spleen cells (3, 4). Since the heavily irradiated cells are themselves incapable of producing hemolysin-forming cells, these observations suggest that the heavily irradiated cells facilitate hemolysin-forming cell responses by small numbers of normal cells. This facilitative function could be mediated either by the production of a conditioning factor or, alternatively, by a mechanism requiring relatively close cell–cell interaction.

In this paper data will be presented showing (i) that the capacity of spleen cells to give rise to hemolysin-forming cells is suppressed by doses of irradiation known to inhibit the capacity of cells to proliferate extensively, (ii) that suppression of the facilitative function of heavily irradiated cells requires much higher doses of irradiation, and (iii) that hemolysin-forming cell responses are generated under experimental conditions allowing close cell–cell interaction between normal and heavily irradiated spleen cells.

[1] This work was supported by the Medical Research Council of Canada (Grant MA-1609) and the Ontario Cancer Treatment and Research Foundation (Project No. 203).

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Materials and Methods. Spleen cells were obtained from 10–12-week-old inbred CBA/J and (C3H/HeNOci × C57BL/60ci)F_1 (designated as B6C3F_1 below) mice. Cell suspensions were prepared in cold tissue culture medium, similar in composition to that subsequently used for the cell cultures. Irradiation of cells to be used was accomplished either by irradiating living animals or by irradiating appropriate suspensions of spleen cells (1×10^8/ml) in cold tissue culture medium. All irradiation procedures were carried out in a ^{137}Cs irradiator (5). When living mice were irradiated they were killed by cervical dislocation immediately after the completion of irradiation. The cell culture system used was similar to that developed by Marbrook (2). Modifications of this system, as used in our laboratory, have been described previously (3). A further modification consists of adding L-asparagine (20 μg/ml) to the culture medium, since it has been found that L-asparagine increases the number of hemolysin-forming cells produced by a given number of spleen cells. Sheep red blood cells (SRBC) were obtained by weekly bleedings of the same sheep and stored in citrate solution. The SRBC were washed three times in phosphate buffered saline before they were added to the cultures. Plaque-forming cells (PFC) were assayed according to the method described by Jerne *et al.* (6). Assays were carried out on the fourth day of culture, because the peak PFC response occurs at this time (4).

Results. The first group of experiments was designed to determine the radiation survival of the capacity of CBA mouse spleen cells to produce PFC in culture. In some of the experiments 2×10^7 spleen cells, obtained either from mice or cell suspensions given in-

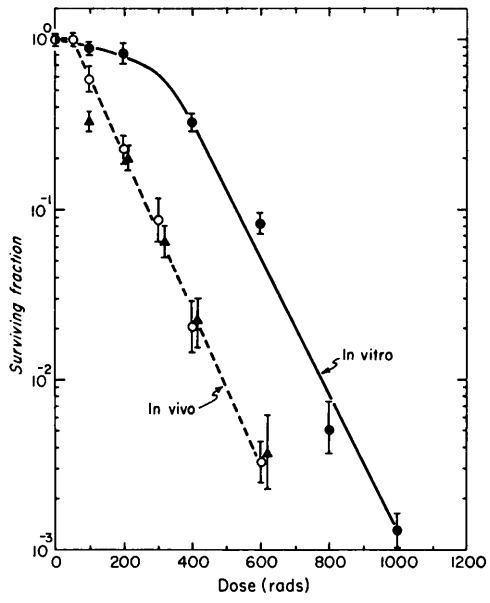

FIG. 1. Radiation survival curves for the PFC response produced by irradiated CBA spleen cells in culture. Spleen cells were cultured after varying doses of irradiation were given to either living animals (---) or cells in suspension (—). The radiation survival of cells from irradiated animals, *i.e.*, 2×10^7 spleen cells cultured alone (○); or 2×10^6 spleen cells (▲) cultured with 2×10^7 heavily irradiated spleen cells is similar, having a D_0 of 95 rads. The D_0 for cells irradiated in suspension is 105 rads. The bars indicate 1 standard error. The larger shoulder in the curve obtained for cells irradiated *in vitro* is similar to that found for hematopoietic stem cells by McCulloch and Till (15).

creasing doses of irradiation were cultured with 2×10^6 SRBC. In separate experiments 2×10^6 spleen cells obtained from mice given varying doses of irradiation were cultured with 2×10^7 heavily irradiated (2000 rads) spleen cells and 2×10^6 SRBC. Control cultures contained the same numbers of spleen cells as described above, but SRBC were not added. Responses in these cultures were considered to represent "background" activity and were subtracted from the total response in cultures containing SRBC. The results are plotted as a survival curve in Fig. 1. The survival curve for cells irradiated in suspension (*in vitro*) has a larger shoulder than that for cells obtained from irradiated mice (*in vivo*), but the slopes of the exponential portions of the curves are similar. The D_0 (the dose of irradiation required to reduce PFC production to 37% of its former level on the exponential portion of the survival curve) for cells irradiated in suspension is 105 rads, while that for cells obtained from irradiated animals is 95 rads.

Small numbers of normal spleen cells unable to give rise to PFC by themselves will be able to do so in the presence of a large number of heavily irradiated cells (3, 4). The following experiments were designed to determine the radiation survival of the capacity of heavily irradiated cells to facilitate PFC production. CBA spleen cells were irradiated in suspension with doses in excess of 1000 rads. These doses were chosen because they are known to completely suppress PFC formation by the irradiated cells (see Fig. 1. above). Suspensions of 2×10^7 irradiated cells, 2×10^6 normal cells and 2×10^6 SR BC were cultured and assayed for PFC on the fourth day. Control cultures containing only

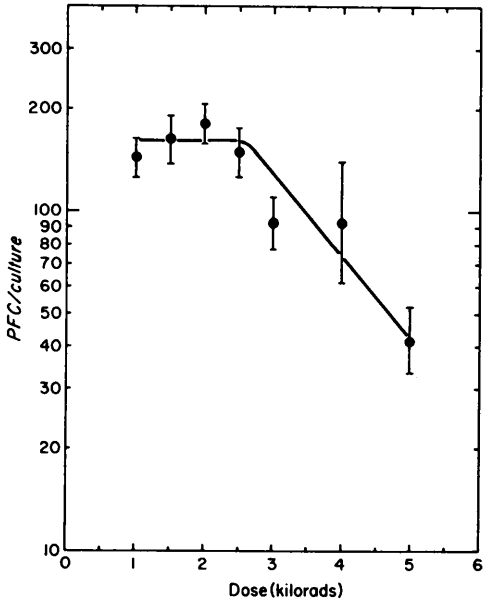

FIG. 2. Radiation survival of the capacity of heavily irradiated CBA spleen cells to facilitate PFC responses in culture by a small number of unirradiated spleen cells. Suspensions of spleen cells were irradiated and 2×10^7 irradiated spleen cells were cultured with 2×10^6 unirradiated spleen cells and 2×10^6 SRBC. The bars indicate 1 standard error.

2×10^6 normal spleen cells and 2×10^6 SRBC did not give rise to significant numbers of PFC. However, 2×10^6 normal cells gave rise to PFC when cultured in the presence of the irradiated cells. Doses of irradiation in excess of 2.5 krads were necessary to suppress the capacity of the heavily irradiated cells to facilitate PFC formation by the normal spleen cell population (Fig. 2).

One way in which heavily irradiated spleen cells may facilitate the production of PFC by small numbers of normal cells is to provide a conditioning factor in the medium. Two experimental designs were used to test this possibility: (i) heavily irradiated cells, mixed with SRBC or alone, were maintained in culture for periods ranging from 24 to 96 hr, and the medium then used for culturing small numbers of normal cells with or without the addition of fresh, heavily irradiated cells and SRBC, and (ii) culture chambers were constructed using two inner chambers. The lower end of the innermost chamber was covered with a Millipore filter having a pore size of 0.1 μ. The heavily irradiated cells were placed in the innermost chamber on the Millipore membrane, and a small number of normal cells was placed in the middle chamber on the dialysis membrane. SRBC were added to either one or both of the chambers. Thus, the irradiated and normal cells were separated only by a Millipore membrane. Neither of the above methods was successful, *i.e.*, the small number of normal spleen cells did not produce PFC unless they were mixed with the heavily irradiated cells. Thus, these methods failed to demonstrate the provision of a conditioning factor by heavily irradiated cells.

An alternative explanation for the facilitative function of heavily irradiated spleen cells is that a cell–cell interaction may be required between irradiated and normal cells. If such an interaction takes place, it should be possible to test its frequency in limiting dilution experiments, by keeping the number of normal (responding) cells constant while varying the number of heavily irradiated cells over a wide range. Cultures in which at least one interaction takes place should be positive for PFC, while those in which there is no interaction should be negative. In separate experiments (unpublished) it has been found that 10^7 $B6C3F_1$ spleen cells fail to give rise to PFC when cultured alone, but give rise to large numbers of PFC when cultured with heavily irradiated CBA spleen cells. Thus, by themselves, $B6C3F_1$ cells may be considered to be rich in responding cells, but poor in cells having a facilitative function. Therefore, in these experiments, heavily irradiated CBA cells varying in number from 1×10^6 to 20×10^6/culture were mixed with 10^7 $B6C3F_1$ spleen cells and 2×10^6 SRBC. PFC assays were carried out on the fourth day of culture. The percentage of cultures positive for PFC in each group of cultures containing a given number of irradiated cells was calculated and plotted as shown in Fig. 3. The curved line represents the theoretical limiting dilution curve, calculated from Poisson statistics, while the solid circles represent the experimental data obtained. The number of irradiated CBA cells required to give 63% positive cultures was approximately 5×10^6/culture, *i.e.*, under these experimental conditions 10^7 $B6C3F_1$ cells did not give

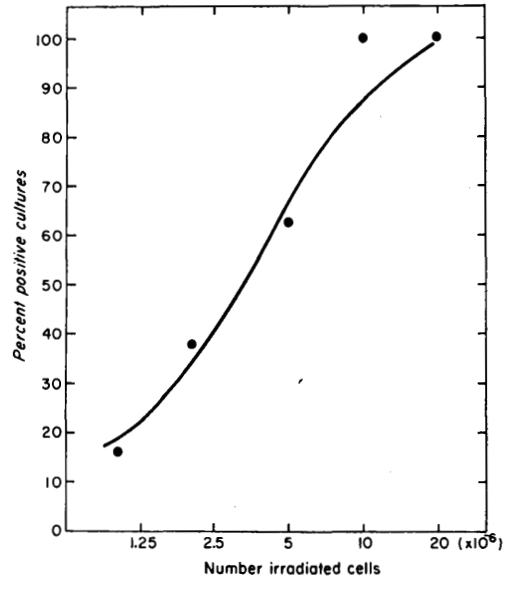

FIG. 3. Relationship between the percentage of "positive" cultures containing PFC to SRBC (●) and the number of heavily irradiated spleen cells per culture: (—), calculated limiting dilution curve, assuming one interaction per 5×10^6 irradiated cells.

rise to PFC unless at least 5×10^6 irradiated CBA spleen cells were also present. Thus, it may be concluded that 5×10^6 irradiated CBA cells contain sufficient facilitating cells to provide at least one interaction between the facilitating cells in the CBA population and the responding cells in the $B6C3F_1$ population.

In addition to the spleen, several other sources of cells were tested for their ability to facilitate PFC production by small numbers of normal spleen cells. These sources included isogeneic lymph nodes, thymus, bone marrow, and L-strain fibroblasts. The latter three, whether irradiated or not, did not facilitate PFC production by spleen cells. Thus, only the spleen and lymph nodes contained cells capable of facilitating the production of PFC.

Discussion. The results of the radiation experiments show that the D_0 is 105 rads for cells irradiated in suspension and 95 rads for cells obtained from mice irradiated during life. These two values are probably not significantly different from each other. Furthermore, they are probably not significantly higher than the value of 80 rads, reported by Kennedy *et al.* (7), obtained by injecting SRBC into the intact animal, or of 70 rads, obtained by Makinodan *et al.* (8), when spleen cells were transplanted into irradiated mice and tested for their capacity to produce hemagglutinin against SRBC. It is generally accepted that an observed D_0 value in this range for mammalian cells represents injury to the capacity of cells to proliferate extensively (9). Since, in the intact animal, precursors of PFC proliferate after exposure to antigen to give rise to PFC (10), the radiation survival data obtained for cells giving rise to PFC in culture suggests that the function injured by radiation may be the capacity of the precursor cells to proliferate in response to antigenic stimulation.

It has been previously reported (3, 4) that cells which have been heavily irradiated, and therefore are incapable of extensive proliferation, can facilitate the production of PFC by a spleen cell population which is too small to give rise to PFC by itself. The present experiments establish that doses in excess of 2.5 krads are required to diminish this facilitative function. Although it is possible that these cells may undergo one or two abortive divisions, we would suggest that they perform their facilitative function by some mechanism other than proliferation.

Experiments designed to detect a conditioning factor in the medium in which heavily irradiated cells were cultured did not detect a conditioning effect, but mixtures of normal and heavily irradiated cells regularly produce PFC responses regardless of whether the cells were cultured in fresh medium or in "conditioned" medium. The requirement for normal and heavily irradiated cells to be mixed is in keeping with the view that relatively close cell–cell interaction is required for production of PFC responses.

There is ample evidence from experiments in intact mice for thymus–marrow interaction during hemolysin responses to SRBC (11). In thymus–marrow synergism both populations of cells are known to proliferate during the response (12). It is likely, on the basis of the experiments in intact animals, that thymus-derived and marrow-derived cells are also present in the spleen cells used for culture. It is reasonable to assume that both of these types of cells undergo proliferation in culture as they do in intact animals. However, in the culture experiments reported here a class of cells was detected which can perform its function—the facilitation of PFC production—in spite of having lost the capacity for extensive proliferation. Such cells could be detected in heavily irradiated spleen and lymph nodes but not in thymus or bone marrow. Thus, the class of cells found in the spleen and lymph nodes is likely to be different from the classes derived from thymus or bone marrow in thymus–marrow synergism experiments in the intact animal.

Recently Mosier (13) has described synergism between two populations of spleen cells, only one of which has the capacity to adhere to plastic. When adherent and nonadherent classes of cells are mixed they give rise to PFC formation in the culture system described by Mishell and Dutton (1). The adherent population can still support PFC production by nonadherent cells after exposure

to 1000 rads (14). Thus, both the Mishell and Dutton and the Marbrook culture systems require the presence of a class of cells capable of facilitating PFC production by a separate class of cells. The class of cells exhibiting the facilitative function can perform this function without requiring a capacity for extensive proliferation. It remains to be seen whether or not this class of cells detectable in culture systems also has a function under physiological conditions in the intact animal.

Summary. Numbers of unimmunized CBA spleen cells, too small to give a PFC response in culture by themselves, can produce PFC if they are cultured in the presence of a large number of heavily irradiated cells. The survival after irradiation of the PFC response by normal spleen cells has a D_0 of 95–105 rads. In contrast, inhibition of the capacity of heavily irradiated spleen cells to facilitate PFC production by normal cells requires doses greater than 2.5 krads. Attempts to demonstrate the provision of a conditioning factor by the heavily irradiated cells were not successful. However, mixtures of normal and heavily irradiated cells in limiting dilution did result in PFC production. These results are in keeping with the hypothesis that cell—cell interaction between the two cell populations is required for generation of PFC responses in culture. Since it is known that in thymus–marrow synergism in intact animals proliferation of both the thymus- and marrow-derived cell populations is required for PFC production, it is suggested that in culture a third, nonproliferating class of cells is also required. This class of cells can be detected in the spleen and lymph nodes but not in the thymus or bone marrow.

We thank Patricia Lee, Sandra Coughlin, Penelope Thompson, and Ted Fromson for excellent technical assistance, and our colleagues at the Ontario Cancer Institute for advice and criticism.

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Received Oct. 14, 1969. P.S.E.B.M., 1970, Vol. 133.