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Effects of ionizing radiation on the reaggregation of embryonic mouse brain cells†

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1. Introduction

In this study we have investigated some effects of radiation on the ability of dissociated embryonic mouse brain cells to form aggregates *in vitro*. Such aggregation of brain cells represents a process involving a high degree of cellular interaction and recognition. Although the mechanism of such cell recognition is unknown, it has been shown for mouse brain that cells from isocortex and hippocampus will reaggregate *in vitro* to form histotypic structures reminiscent of the *in vivo* mouse brain (DeLong 1970 a,). Also, in such dissociated and reaggregated embryonic mouse brain cells, the levels of choline acetyltransferase, acetylcholinesterase, and glutamate decarboxylase increase similarly to those found in the embryonic mouse brain *in vivo* (Seeds 1971, Seeds and Gilman 1971). At longer periods of reaggregation *in vitro*, such reaggregates develop synaptic connections (Seeds and Vatter 1971).

This relatively novel *in vitro* system of culturing brain cells might therefore provide information concerning the effects of radiation on some cellular activities that do not explicitly involve cell proliferation.

2. Materials and methods

2.1. *Animals and cell culture procedures*

Foetuses were taken from pregnant C57B1/6J mice (Jackson Laboratories, Bar Harbor, Maine). The gestation time is 19–21 days and females estimated to be 17–18 days pregnant were killed by cervical dislocation. The abdomen was opened aseptically, and the foetuses removed to sterile Petri dishes. They were then decapitated, the skull opened, and the meninges removed. The cerebral hemispheres were dissected and placed into a small volume of sterile calcium–magnesium-free phosphate-buffered isotonic saline (CMF) containing 0·1 per cent glucose. The CMF also contained 2·5 μg/ml of DNAase (Worthington Biochemicals) to prevent random clumping of dissociated cells while in CMF. The cerebral hemispheres in CMF were placed into a modified 50 ml syringe apparatus and gently forced through a stainless-steel mesh of 240 μm diameter. This initial suspension was collected and allowed to flow by gravity through a mesh 140 μm in diameter and then through one of 60 μm. The filtrate

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was collected and centrifuged at 156 g for 3 min to pellet the cells. The pellet

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was then resuspended in Eagle's basic medium with added 0·1 per cent glutamine, 2·5 μg/ml of DNAase, 10 per cent horse serum, and supplemental glucose was added as 5 per cent dextrose in saline to a total glucose concentration of 600 mg/100 ml. Cell counts were performed with a haemocytometer, and the cell concentration was adjusted to $1{\cdot}0 \times 10^6$ cells per ml. Cells were placed into small 1 cm diameter vials at the above seeding concentration in a total volume of 0·5 ml. Cultures were gassed with 4 per cent carbon dioxide in air, placed on a rotating shaker, and rotated at 70 r.p.m. using a gyrorotary diameter of 2·25 cm at 37°c. Cultures were assayed for aggregate development after 4 days in rotation culture. Photographs of the aggregates were taken using an inverted microscope at a magnification of 50×. As the aggregates are usually spherical, their total volume may be calculated from the formula of a sphere, or in special cases, from the formula of an ellipsoid.

2.2. *Histology*

As the aggregates formed are relatively small (0·1 to 0·3 mm diameter), the formed aggregates are trapped in a plasma clot for tissue processing (Shands 1968). This plasma clot containing the aggregates is then fixed in buffered formalin and processed with routine techniques with embedding in paraffin. Sections 10 μm thick were made and stained with haematoxylin and eosin for estimation of the cellularity of the aggregates.

2.3. *Irradiation procedures*

Irradiations were performed on the dissociated cells after they had been resuspended in medium, using a 1500 Ci ^{60}Co gamma-ray source. Cells were irradiated in glass vials at a distance of 70 cm from the source. At 45 cm from the source, a 5 mm-thick lucite shield was placed to absorb any secondary electron production. Dose–rates at 70 cm were approximately 100 R/min over the duration of the experiments. Exposures were measured using a Victoreen 250 R probe in conjunction with a Victoreen R-meter.

3. Results and discussion

In the initial dissociated cell suspension, several types of cells may be found. In figure 1 is presented a size distribution of the relative percentage of cells at different sizes. The distribution shows that most have an average diameter of about 10 μm, but there is a skewness towards larger cells. The maximum diameter of cells obtained was approximately 25 μm. Our size distribution of cerebral hemisphere cells agrees quite well with that obtained in rabbit brain cerebral cortical cells by Hamburger, Eriksson and Norby (1971). In terms of cell morphology, two sub-divisions may be made. There is a group of cells that, immediately after dissociation, are seen to take up the vital stain, trypan blue. These cells (TB+) have diameters of 13·5 μm or greater. No cells smaller than 13·5 μm diameters were observed to show trypan-blue positivity (TB− cells). The sharp demarcation of these two populations in terms of trypan-blue uptake suggests that they represent two different cell-types and do not simply reflect membrane damage due to the mechanical disruption. Histology performed on the cells greater than 13·5 μm showed cell morphology

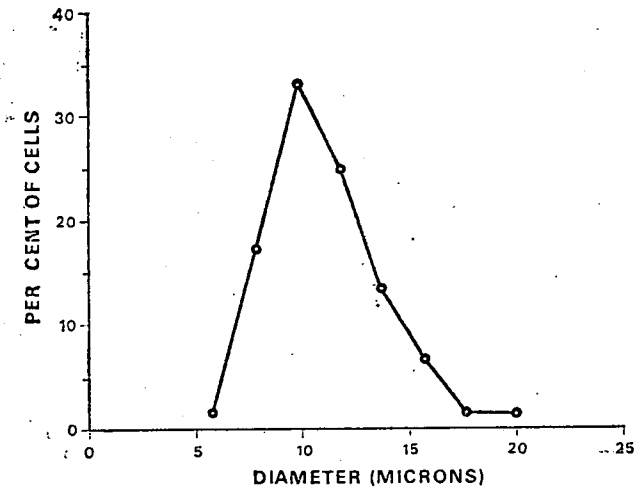


Figure 1. Size distribution of cell suspension obtained from embryonic mouse brain, prepared in calcium–magnesium-free phosphate-buffered isotonic saline.

presumed to be that of large neurons and was very similar to large neuronal cells described by Varon and Raiborn (1969) for chick embryo telencephalon. The presence of a TB^+ cell population does imply a fragile or permeable membrane, and interestingly, our early attempts at embryonic brain dissociation involved the use of the enzyme trypsin, which completely removed this TB^+ cell population. At all enzyme concentrations used (1·0, 0·3, 0·15, and 0·001 per cent), there was total loss of the TB^+ cells with diameters greater than about 15 μm, and it is only with the mechanical dissociation that this population of cells was preserved. This finding raises questions about the results of investigators who have studied brain-cell reaggregation using relatively high concentrations of trypsin (i.e. DeLong 1970 a,). It is probable that the smaller TB^- cells comprise at least two sub-divisions—small neuronal and neuroglial cells. The TB^- cells are small, dense, and it is difficult to distinguish nuclear characteristics. It is likely that mesodermal and ependymal cells are also included in the heterogeneous initial cell dissociate. The red blood-cell contamination of the dissociate was about 6 per cent in all experiments (i.e. 5 red blood cells per 100 brain cells) and this was not significantly changed by the centrifugation and pelleting of the cell suspension. A typical cell yield was about $1{\cdot}2 \times 10^6$ cells per embryonic brain (i.e. two cerebral hemispheres). This is about $4{\cdot}0 \times 10^4$ cells per mg of brain. Of the total yield, about 23 per cent are the TB^+ cells.

In figure 2 are two typical reaggregates at 4 days after seeding in culture. In figure 3 shows a haematoxylin- and eosin-stained histological section of a reaggregate. From the histological preparation, it is possible to estimate the degree of cellularity of the aggregates and to determine the percentage of the reaggregate volume devoted to cells and to intracellular matrix. We have estimated that about 50 per cent is composed of cells. For the determination of a dose–response curve for radiation effects then, the following data were used. As the originally seeded number of cells is known, and as the number and total

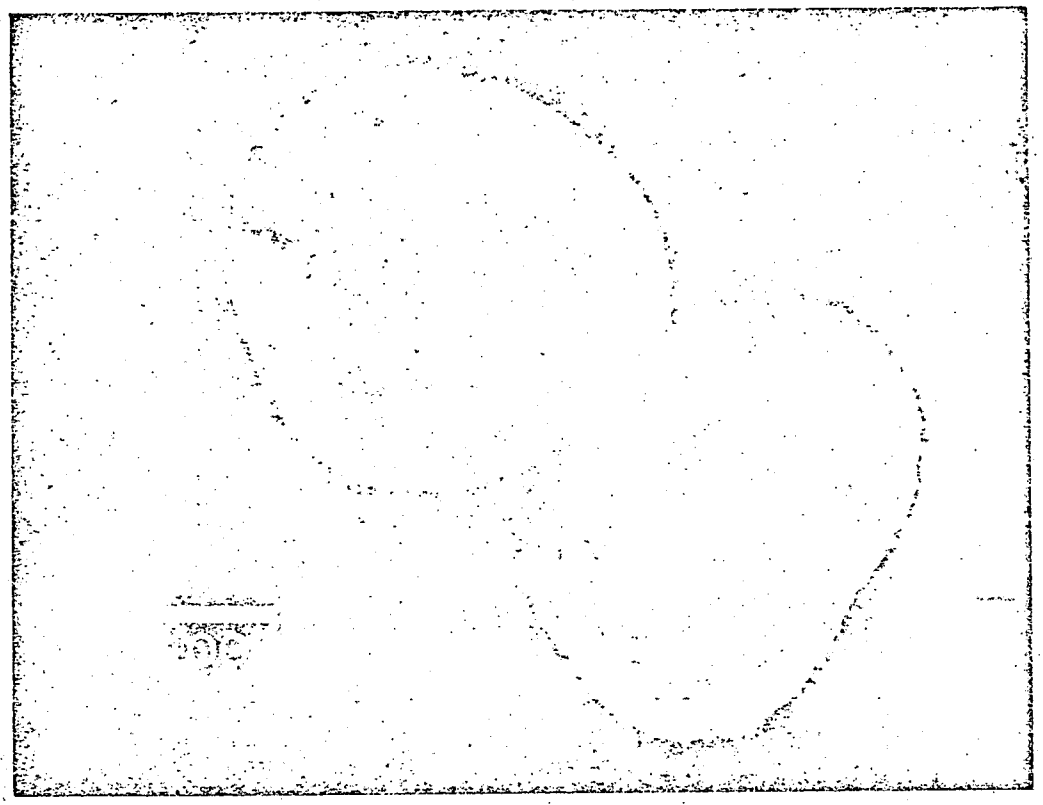
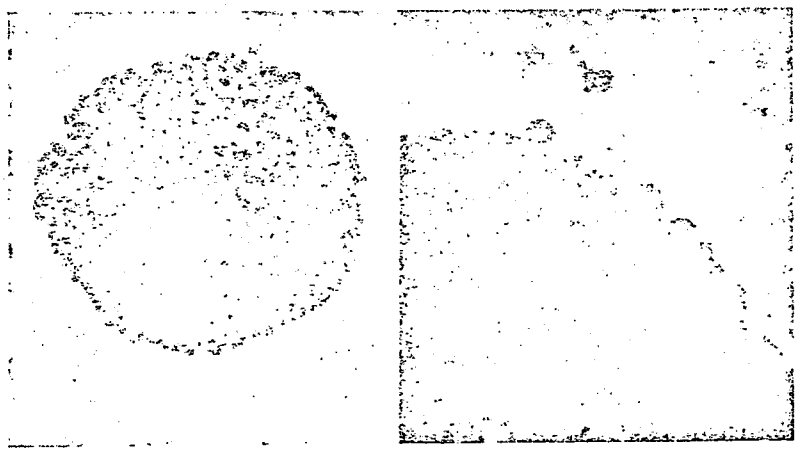


Figure 2. Two reaggregates from dissociated 17-day-old mouse embryonic brain after 4 days in gyrorotary suspension culture. Phase contrast photograph. The insert bar indicates 100 μm length.



(*a*) (*b*)

Figure 3. Internal structure of a mouse cerebral hemisphere reaggregate after 4 days in gyrorotary suspension culture. Stained with haematoxylin and eosin, paraffin section 10 μm thick. (*a*) illustrates low-power structure (× 100); (*b*) a higher-power photograph of an edge of the aggregate (× 100).

volume of the reaggregates may be estimated, and as the degree of cellularity of the reaggregates may be determined histologically, we are then able to estimate the percent of recovery of the originally seeded cells in formed reaggregates. Such a percent recovery response is shown in figure 4. The dose–response curve has an extrapolation number of 1·06 and a D_0 of 153·6 R, using conventional survival curve parameters. In the table the experimental data used to determine the dose–response curve are presented. It definitely appears that some component of the reaggregation process has been damaged by radiation in a dose-dependent manner.

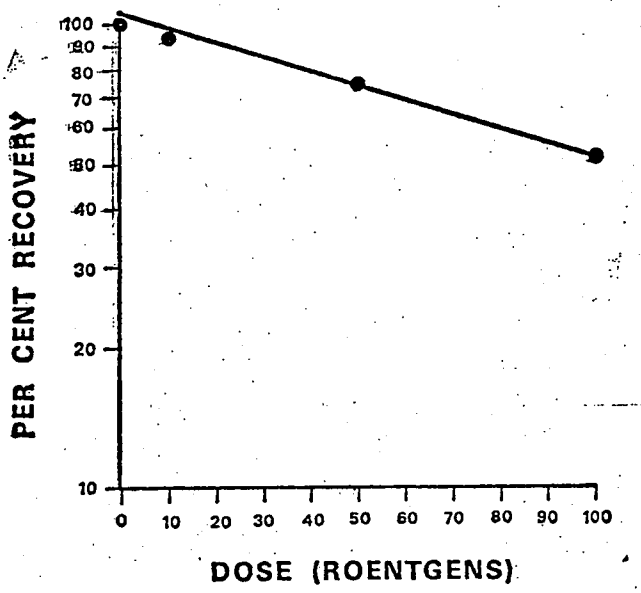


Figure 4. Percent recovery of originally seeded mouse embryonic brain cells in formed aggregates at 4 days in gyrorotary suspension culture after 60Co gamma-irradiation.

Dose (Roentgens)	Number of cultures	Cell recovery (percentage of control values)†
Control	10	100 ± 4·2
10	11	94 ± 7·3
50	13	74 ± 9·3
100	9	52 ± 14·4

† Standard error of the mean.

Changes in the percent recovery of cultured dissociated embryonic neural cells in aggregates at 4 days after ^{60}Co gamma ray irradiation.

It must be emphasized that although a radiation-dependent phenomenon occurs with this type of cell culture, the mechanism through which the effect is mediated is not known. Several possible routes may be suggested, as for example direct cell-killing, although at the low doses used in these experiments,

this appears unlikely. As the phenomenon of reaggregation is thought to be dependent on membrane integrity, and in particular the presence of certain glycoprotein (Richmond, Glaeser and Todd 1968), or acid mucopolysaccharide (Pessac and Defendi 1972) moieties at the cell surface, possibly the radiation is interfering in some manner with the biosynthesis of materials needed for proper cell interaction.

With regard to irradiations of mouse embryos *in vivo* at comparable stages of gestation, Dekaban (1969) has reported that 200 R of x-rays produced only very minor architectural changes in mouse cerebral cortex when foetuses were irradiated at a stage of gestation comparable to the stage used in this study for reaggregation. In conclusion, it would appear that the reaggregation of mammalian tissues may be a worthwhile system for investigation of radiation effects.

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