Effect of Ionizing Irradiation on Susceptibility of McCoy Cell Cultures to *Chlamydia trachomatis*

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The effect of graded doses of irradiation (cobalt-60) on the morphology of McCoy cells was analyzed, and 4,000 to 5,000 r was selected as a satisfactory dose for production of giant cells. The susceptibility of radiation-induced giant cells to chlamydial infection was compared with that of nonirradiated cells by using three strains of *Chlamydia trachomatis* and one of C. psittaci. Monolayers of giant cells were more susceptible than normal McCoy cells as indicated by (i) greater numbers of inclusions (four- to eightfold) per unit area of monolayer, (ii) larger inclusions (fourfold greater in area), (iii) higher infective titers (1 log or more greater) of harvested cells, and (iv) greater ease of promoting a second cycle of growth. Graded doses of irradiation were applied also to mouse fibroblast (L) cells, and a similar increase in susceptibility to chlamydial infection was noted. It is concluded that giant cells produced by irradiation possess advantages over nonirradiated cells in culture for growth of *Chlamydia*.

Strains of *Chlamydia trachomatis* from various sources will infect many types of cell cultures, but progressive multiplication beyond the first developmental cycle is minimal or absent with most strains. The initial growth can, however, be detected by observation of stained intracytoplasmic microcolonies (inclusions) of the microorganisms, and passage can be effected if special methods are used (8). Preliminary investigations (unpublished) indicated that the McCoy cell line (5) was as susceptible as any of several cell systems tested, and possibly more so than some, to yolk sac-established chlamydial strains. We noted that a small proportion of McCoy cells, several times larger than the normal cell, and often containing multiple nuclei, occurred among the usual mononucleated polygonal cells in an incidence estimated at one in several hundred. We had the distinct impression that a greater proportion of these (giant) cells in infected cultures contained inclusions than did the smaller cells, suggesting a greater susceptibility of the former. Therefore we undertook a test of the susceptibility of cultures composed of irradiation-produced giant cells. Pomerat et al. (18) produced giant cells by irradiation of McCoy cell cultures, and Fernandez (5) demonstrated the greater susceptibility of these giant cells to bluetongue virus.

We describe here the laboratory-adduced evidence for the greater susceptibility to chlamydial infection of McCoy line and L929 (mouse fibroblast) line giant cells induced by ionizing irradiation, in comparison to nonirradiated cells.

**MATERIALS AND METHODS**

*Chlamydial strains.* Infected yolk sac suspensions, prepared by standard methods in sucrose-phosphate solution (0.2 M sucrose in 0.02 M phosphate buffer, pH 7.0) were stored at –65 C. *C. trachomatis*, strains Cal-1, MRC-1/G, and Mopn (mouse pneumonitis) and C. psittaci, strain Felpn (feline pneumonitis), described elsewhere (9), were used.

**McCoy cell line.** The origin and description of the McCoy cell line have not been adequately documented. Early references to its use (18, 20) indicate that it originated in C. M. Pomerat's laboratory. It has been used by a number of persons interested in *Chlamydia* (17), and cultures have frequently been exchanged. Although originally regarded as a human cell derived from synovial fluid, evidence has accumulated from several laboratories that cultures now labeled McCoy are composed of mouse cells. One published reference (11) raises this possibility, and
an attempt has been made here to bring together items of evidence for the McCoy line being a mouse cell, derived by personal communication from the sources indicated: (i) karyotype examination (O. A. Holtermann); (ii) not infected by poliovirus type 1 (G. E. Kenny); (iii) McCoy cells were susceptible to action of macrophages from mice immunized with L (C57 mouse) cells (G. E. Kenny); (iv) mixed agglutination tests performed in Dr. Coomb’s laboratory indicated mouse (G. E. Kenny); (v) a positive immune adherence test with antisem prepared in (Balb C × C57 Bl) F1 against C3H (H-2K) mice having anti-H-2.11 specificity (K. W. Sell); (vi) mouse interferon reduced chlamydial growth in McCoy cells (F. B. Gordon).

Cell cultures. Stock cultures of the McCoy cell line were maintained in T-flasks or prescription bottles in Eagle’s minimal medium without antibiotics plus 1% glutamate and 10% horse serum (MEM, HoS). at 37°C. Mouse fibroblast cells (L-229) were grown similarly but with 10% inactivated calf serum. Suspending or irradiated cells in appropriate dilutions in the same medium were placed in flat-bottomed tubes (1.5 by 12 cm) containing 12-mm circular cover slips, to provide confluent cell monolayers 48 hr later. In the case of the irradiated cells which no longer multiplied, the monolayer was formed by migration, flattening, and increase in size of individual cells.

Irradiation of cell cultures. Stock cell cultures or tubed suspensions of cells from stock cultures were exposed to gamma radiation from a cobalt-60 source in a Theratron Jr. (General Electric Co., Inc.), and for this assistance we are grateful to personnel of the Naval Hospital, National Naval Medical Center. Dosage rates varied from 40.8 to 115 r per min. After exposure to irradiation, cells were reseeded into culture bottles and allowed to attach, and one or more changes of medium followed in the course of a few days, which served to remove the cells that did not survive the irradiation. After 5 to 12 days, the surviving cells were seeded onto cover slips as described above and used 2 to 4 days later.

Inoculation of cultures. Cell monolayers formed on cover slips in flat-bottomed tubes were inoculated by replacement of the medium with 0.5 ml of appropriate dilutions of stored infectious yolk sac preparations. The complete medium used was MEM, HoS, 10 to which glucose had been added to provide a final concentration of 30 μM per ml. The inoculated cultures were centrifuged horizontally at 1,600 to 1,800 x g for 1 hr at 20°C and incubated at 35°C. Both addition of glucose (Vedros and Gordon, 1963, Bacteriol. Proc., p. 134 and centrifugation (22) have been shown to increase infectivity in this type of agent-host cell system. When heavy inocula were used, involving undesirable concentrations of yolk sac material, the inoculum was replaced with complete medium at 2 hr or later after the termination of centrifugation.

Examination of cultures. At various intervals after inoculation, the cover slip monolayers were washed with a balanced salt solution and fixed with methyl alcohol. Staining was with a 2.5% iodine solution for enumerating the glycogen-containing inclusions, or by May-Greenwald-Giemsa (MGG) for examining the cells (430 x) and for observing the nonglycogen-forming inclusions (Felp). Cell measurements were made with a calibrated ocular micrometer.

Iodine-staining inclusions were counted at a magnification of 200x in 50 or more microscopic fields at various locations over the cover slip. By use of the appropriate factor, an estimate was made of the number of inclusion-forming units that had infected the entire cover slip.

Titrations in chick embryos. Titrations were performed in chick embryos by standard methods with a volume of 0.5 ml for inoculation of the yolk sac of 6- or 7-day-old embryos.

RESULTS

Morphological observations on irradiated cells. Monolayer cultures of McCoy cells established on cover slips were exposed in groups to varying doses of irradiation. The monolayer of one tube in each group was fixed at each of the time periods indicated in Table 1 and stained with MGG. On each cover slip, 25 cells were selected by a procedure designed to insure randomness, the number of nuclei per cell was counted, and the cells were measured along their longest axis (L) and along their greatest dimension (W) at right angles to L. The morphology of normal and irradiated cells is illustrated in Fig. 1 and 2.

A clear index of effect of ionizing irradiation on the cells is the appearance of irregularly-shaped and multiple nuclei (Fig. 2). Table 1 records the number of cells, of each group of 25 observed, with more than one nucleus and the average number of nuclei per cells. (Large lobes of nuclei, almost completely separated from other nuclear masses were regarded as separate nuclei.) Estimates of mean cell areas (A) of selected monolayers (Table 2) were made by using the formula, A = 0.643 LW.

| Irradiation dose (r) | Type of count | Days after irradiation |
|---------------------|---------------|-----------------------|
|                     |               | 1   3   6   9   12   16     |
| 0                   | a              | 1   0   0   0   2   0       |
|                     | b              | 1.0 1.0 1.0 1.0 1.1 1.0     |
| 500                 | a              | 2   2   1   1   6   6       |
|                     | b              | 1.1 1.1 1.0 1.1 1.4 1.4     |
| 1,000               | a              | 2   10  10  11  11  10      |
|                     | b              | 1.1 1.9 1.6 1.8 1.8 2.1     |
| 2,000               | a              | 0   15  11  18  18  16      |
|                     | b              | 1.0 2.9 3.0 3.6 3.1 4.6     |
| 4,000               | a              | 0   13  13  20  19  20      |
|                     | b              | 1.0 2.5 5.7 5.3 9.4 7.7     |
| 7,000               | a              | 2   6   10  21  23  16      |
|                     | b              | 1.1 1.8 1.6 9.7 7.9 7.8     |

a, Number of cells (per 25) with multiple nuclei; b, average number of nuclei per cell.
TABLE 2. Mean areas (μm²) of McCoy cells at intervals after irradiation

| Irradiation dose (r) | Days after irradiation |
|----------------------|------------------------|
|                      | 1          | 3          | 6          | 12         |
| 0                    | 161 ± 10.1 | 109 ± 7.3  | 144 ± 8.9  |
| 500                  | 118 ± 9.6  | 415 ± 36.5 | 186 ± 24.5 |
| 1,000                | 256 ± 26.1 | 440 ± 49.2 | 608 ± 24.5 |
| 2,000                | 210 ± 17.3 | 884 ± 73.1 | 114.9 ± 26.1|
| 4,000                | 233 ± 19.6 | 1,305 ± 151.1 | 1,544 ± 379 |
| 7,000                | 260 ± 17.5 | 486 ± 64.6 | 1,590 ± 276.7 |

*Each figure was derived from measurements on 25 cells.

Minimal morphologic changes were produced by 500 r and were clearly detected only as an effect on the nuclei observed first on day 12 (Table 1). Higher radiation dosages produced noticeable nuclear effects on day 3, which became greater during the following days, especially with the two largest radiation doses. Increased dimensions of the cells (Table 2) were not clearly detected until doses of 2,000 r or greater were used, but at 6 days or more after receiving 4,000 r the surviving cells had areas considerably greater than the average nonirradiated cell. There was evidently some lag in this effect with the highest radiation dose, 7,000 r. Comparison of average cell areas at the time monolayers are used, i.e., 1 to 3 days after setting of nonirradiated cells, and 6 to 8 days after irradiation, indicated approximately a 10-fold difference in mean cell area in the two types of monolayers (Table 2).

In the following experiments, the irradiation dose was 5,000 r, and cells were inoculated at varying periods from 7 to 20 days after exposure. There was no apparent advantage in using the longer periods, and we now routinely use cells at 7 days after irradiation with 5,000 or 4,000 r.

Susceptibility to infection of irradiated and nonirradiated cells; numbers of inclusions. Table 3 summarizes a series of experiments in which chlamydial strains were inoculated onto irradiated and nonirradiated monolayers and the results were compared by counting the number of inclusions produced in sampled areas of replicate cover slips of each after fixation at 48 hr. Inclusions seen at this interval are the results of a first cycle of infection, i.e., are present only in cells initially infected. The ratios of inclusion counts in irra-
Table 3. Susceptibility of irradiated (5,000 r) and nonirradiated McCoy cell monolayers to infection with C. trachomatis

| Chlamydial strain | Interval to cell use after irradiation (days) | Dilution of inoculum (negative log$_{10}$) | Mean no. of inclusions per cover slip | Irradiated cells (IR) | Non irradiated cells (N) | IR/N |
|-------------------|---------------------------------------------|------------------------------------------|-----------------------------------|----------------------|-------------------------|------|
| Cal-1             | 7                                           | 6,357                                    | 1,132                             | 5.6                  |                         |      |
|                   | 4                                           | 587 ± 58                                 | 86 ± 15                           | 6.8                  |                         |      |
|                   | 5                                           | 52                                       | 4                                 |                       |                         |      |
| Cal-1             | 8                                           | 1,960                                    | 441                               | 4.4                  |                         |      |
| Cal-1             | 8                                           | 2,218 ± 85                               | 300 ± 58                          | 7.4                  |                         |      |
| Cal-1             | 8                                           | 3,220 ± 375                              | 517 ± 77                          | 6.2                  |                         |      |
| Cal-1             | 4                                           | 353                                      | 27                                |                       |                         |      |
| Cal-1             | 10                                          | 2,515                                    | 346                               | 7.3                  |                         |      |
| Cal-1             | 10                                          | 2,254                                    | 286                               | 7.9                  |                         |      |
| Cal-1             | 13                                          | 226                                      | 34                                | 6.6                  |                         |      |
| Cal-1             | 3.5                                         | 72                                       | 6                                 |                       |                         |      |
| Cal-1             | 14                                          | 181 ± 27                                 | 37 ± 2                            | 4.9                  |                         |      |
| Cal-1             | 14                                          | 1,117                                    | 147                               | 7.6                  |                         |      |
| MRC-1/G           | 14                                          | 191 ± 21                                 | 42 ± 4                            | 4.5                  |                         |      |
| Mopn              | 13                                          | 4,880                                    | 247                               | 19.8                 |                         |      |
|                   | 5                                           | 832                                      | 23                                |                       |                         |      |
|                   | 6                                           | 103                                      | <12                               |                       |                         |      |

was not sufficiently great to allow accurate quantitative observations with the nonirradiated, uncentrifuged cultures, significant differences are clear whether centrifugation or irradiation is the factor assessed.

Promotion of a second growth cycle. The C. trachomatis strain Cal-1, used in most of these tests, does not ordinarily undergo a second cycle of growth in tube cultures of McCoy cells, irradiated or nonirradiated. One of the methods we have investigated for promoting continuing chlamydial infection in the same cell culture is to suspend the cells of the infected culture near the end of the first developmental cycle and reset these in the same tube. Suspension was accomplished by vigorous shaking on a Vortex Genie (Scientific Industries, Inc.). The rationale is to disrupt infected cells by agitation and allow release of infectious particles, which otherwise do not readily escape from the host cell, to initiate a second cycle. Most uninfected cells are not harmed and reestablish a monolayer. This maneuver was applied to the experiment illustrated in Table 6. All tubes received the same inoculum of strain Cal-1. The cells of two were fixed, and those of two others were suspended and reset at each of the three time intervals.

The cultures fixed at 48 hr and later showed inclusion counts greater in irradiated cells, although not to the extent usually seen (two
Table 6. Promotion of a second cycle of chlamydial growth

| Disposition of culture | Inclusion counts |
|------------------------|------------------|
|                        | Irradiated cells | Nonirradiated cells |
| Fixed at 48 hr         | 74               | 35               |
| Fixed at 72 hr         | 42               | 23               |
| Fixed at 96 hr         | 27 <1            |
| Suspended and reset* at 48 hr | 186   | 0               |
| Suspended and reset* at 72 hr | 706   | 0               |
| Suspended and reset* at 96 hr | 550   | 4               |

* Monolayers were fixed 48 hr after resetting.

As previously reported (9), 48 hr is an optimal time for detecting glycogen-positive inclusions, and those identifiable by iodine-staining subsequently decrease in number, as was seen here. No evidence was seen of a spontaneous second cycle of growth. When the cells of 48-, 72-, and 96-hr infected irradiated cultures were suspended, reset, and fixed 48 hr later, it was evident that second cycles of growth occurred. An approximate 10-fold increase is seen in the cultures suspended and reset at 72 hr, assuming the initial 48-hr count represents the total number of inclusions present at 72 hr. Other data also indicate that the 72-hr interval is an optimal time for harvest. It is clear from these results that this method favors a second cycle of growth in irradiated cells, but not in nonirradiated. A second experiment of the same design confirmed this conclusion. This result may be due to a greater fragility of the larger (irradiated) infected cells, resulting in a greater release of infectious particles, as well as to the greater susceptibility of the irradiated cells.

Relative size of inclusions. Figures 3 and 4 show the appearance of inclusions of the MRC-1/G strain in the two types of cells. Measurements were made by means of an ocular micrometer on 25 inclusions chosen at random in each type of monolayer illustrated. Most inclusions were spherical, or nearly so, as reflected in the mean dimensions recorded in Table 7. In the nonirradiated cells, 13 of 25 inclusions had lengths equal to widths. This was true of 7 of 25 inclusions in irradiated cells. Average linear dimensions of inclusions in irradiated cells are seen to be double those of non-irradiated cells.

Infectivity titer of harvest. Several experiments were performed in which three different strains of chlamydiae, including C. psittaci, were used at varying dilutions for inoculation of irradiated and nonirradiated McCoy cultures by the standard procedure. After incubation, harvests were made by resuspending the cells at selected intervals, freezing and thawing, and titrating for infectivity by inoculation of serial 10-fold dilutions into the yolk sac of embryonated eggs. The results are expressed as number of embryo LD₅₀'s per 0.5 ml of cell culture harvest (Table 8). Harvests from infected irradiated cells at the 72-hr (optimal) interval had yolk sac infectivity titers more than 1 log unit greater than those from nonirradiated cells, and the harvests taken at 48 and 96 hr showed similar differences in the same direction. It is clear that the greater number and size of inclusions in irradiated cells is reflected in a greater harvest of infectivity.

DISCUSSION

The ratio of numbers of inclusions in irradiated cultures to those in nonirradiated cultures per unit area of monolayer was usually between 4 and 8. The ratio of numbers of inclusions per cell in the two types of cells was not directly determined but can be estimated from the measurement of areas occupied by individual cells. The figure for area of irra-

Fig. 3. Monolayer of infected (Cal-l), nonirradiated McCoy cells. Arrows indicate inclusions. Methanol fixation 48 hr after inoculation; stained with iodine solution; magnification 230×.
incidence of inclusions per cell in complete monolayers could therefore theoretically be 40 to 80 times greater in irradiated cells than in nonirradiated.

Although the factors responsible for the greater susceptibility are obscure, the physical nature of the large, flat cells resulting from irradiation, is probably significant. The large, flat, nondividing cells obtainable from explants of 4-day chick embryo entoderm (23) were found to be especially suitable for growth of chlamydiae.

Inclusions in irradiated cells were approximately four times greater in area on the average than those in nonirradiated cells, as observed with the microscope, but it is possible that the inclusions were spread out to a greater extent in the giant cells. However, the relative number of inclusions per unit area of monolayer (four- to eightfold difference) does not appear to account entirely for the differences observed in infectivity titers of 72-hr harvests, which were one or more log units greater in irradiated monolayers. It is therefore probable that the individual inclusions of irradiated cells actually do contain greater numbers of infectious particles.

Levine (12) showed that the yield of poliovirus from irradiated primary monkey kidney cells was greater than that from nonirradiated, when both were expressed as yield per cell, and the greater yield in the individual irradiated cells was proportional to the increase in size of the cell. Hsuing (10) and Cieciura et al. (2) have also reported on relationships between viral infection and irradiation of cell cultures. Fernandez (5) found that cultures of giant cells induced by cobalt-60 irradiation of several cell lines, including McCoy, showed earlier or greater cytopathic effects and a higher yield when inoculated with bluetongue virus than did control cultures. He provided evidence that this was correlated with the increased size of the cell rather than with any immediate effect of irradiation. Arita and Matumoto (1) found that replication of measles virus was enhanced in FL cells that had reached certain levels of ultraviolet irradiation, and suggested that this effect is attributable to the failure of the irradiated cells to produce some inhibitory factor such as interferon. They had seen a similar enhancement of viral growth in cells treated with actinomycin D (15). Invasion by chlamydiae results in inhibition of deoxyribonucleic acid (DNA) synthesis in the host cell (3, 19). Whether interference with normal DNA synthesis by another factor (irradiation) (13) is of advantage to chlamydiae in their intracellular growth is not known.

An increased yield of Rickettsia prowazeki...
from irradiated monolayer cultures of chick entodermal cells was observed by Weiss and Dressler (21) early in the infection. This was attributed to a more rapid release of rickettsiae from the cells during lysis after very large doses of irradiation. The spontaneous release of infectious particles from host cells was not assayed in the present experiments, but the observations summarized in Table 6 suggest that irradiated McCoy cells are much more likely to release infectious particles when agitated than are nonirradiated cells.

Nonirradiated McCoy cells have been used successfully for detection of chlamydiae in a laboratory infection (14) and in the eyes of experimentally infected monkeys (7), and the usefulness of irradiated McCoy cells for detection and isolation of chlamydiae in natural human ocular and genital tract infection has been demonstrated in several studies (4, 6, 8, 16). Nonirradiated cells have also been tried with partial success in field studies on trachoma (Gordon et al., unpublished data), but no direct comparisons have been reported between the two types of cells with field specimens.

A distinct advantage of irradiated cells over nonirradiated, readily apparent to the operator who scans the stained cover slip for the detection of infection or counting of inclusions, is the larger size of the inclusions in irradiated cells, greatly facilitating their recognition.

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