EXPERIMENTAL PARASITOLOGY 9, 87–91 (1960)

Radiosensitivity of *Entamoeba histolytica* cysts

Curt R. Schneider[1]

School of Public Health, University of Michigan, Ann Arbor, Michigan

(Submitted for publication, 24 February 1959)

A dose of approximately 8,400 RAD noticeably lowered the number of viable cysts of *E. histolytica* in a clear, saline suspension, as calculated by the Most Probable Number method. Doses of approximately 16,700 RAD and more destroyed more than 99% of viable cysts. The relation between increasing cyst mortality and increasing gamma-ray dosage is represented by a logarithmic straight line.

A difference of 24 hours in age had no demonstrable effect on response of cysts to irradiation.

Cysts of the NRS strain are roughly one-tenth as resistant to gamma rays as are trophozoites of the same strain.

In a previous study (Schneider and Porter, 1960), the radiosensitivity of a non-encysting strain (UC) of *Entamoeba histolytica* was reported. Subcultures of irradiated trophozoites began to display a notable increase in lag phase following exposure to approximately 100,000 RAD (corresponding to 100 ergs per gram) of pure gamma rays. A dose of approximately 195,300 RAD or more resulted in failure of subcultures to grow.

Unsuccessful attempts were made to promote encystation of the UC strain in order to study the radiosensitivity of cysts. Accordingly, a strain of *E. histolytica* (NRS)[2] was obtained which produces cysts in relative abundance in the course of routine culture. The NRS strain is one of Dobell's "*histolytica*-like" species, originally obtained from a macaque (Dobell, 1931). It has been characterized by Chang (1946).

Materials and Methods

The source of gamma rays used in these experiments has been described in another paper (Schneider and Porter, 1960).

NRS amebae were maintained routinely in Ringer-egg-Locke (REL) slants, transferring every 48 hours. Horse serum (0.5 ml per tube) and rice starch[3] were added at the time of each transfer. Cysts were harvested from 72-hour cultures by pooling the contents of eight tubes, centrifuging at 1000–1500 r.p.m. for 4 or 5 minutes, and washing twice or three times in sterile distilled water. Examination of the organisms after this treatment always revealed numerous trophozoites in the process of lysis as well as many cysts which were highly refractile and appeared undamaged. After staining with iodine, most of the cysts were seen to be uninucleated but there was usually a satisfactory number of quadrinucleated cysts as well. Dobell (1928) has stated that only mature (i.e., quadrinucleated) cysts can excyst. Since cyst suspensions were precious and often contained barely enough cysts to work with, the practice of Rees *et al.* (1950) of discarding harvests showing less than 50% quadrinucleated cysts was not followed.

The suspensions were routinely placed in the cold room (4–6°C) overnight and were irradiated on the following day. Prior to irradiation cysts were resuspended in sterile Stone's Locke solution and counted in the hemocytometer. The suspension was ad-

[1] Present address: Division of Medical Sciences, National Academy of Sciences—National Research Council, Washington, D. C. Part of this work was done under a contract with the U. S. Army Medical Department.

[2] Acknowledgment is gratefully offered to Dr. S. L. Chang for making this culture available.

[3] From Stein-Hall Company, New York, N. Y.

justed until six counts gave an average of about 5,000 cysts per milliliter. Actual counts ran from 4,600 to 7,700, but this variation seemed to make little difference in the subsequent calculation of MPN values (see below)

Since determination of the survival of individual irradiated cysts was impractical, the method of Chang and Baxter (1955) for calculating the Most Probable Number (MPN) of survivors was employed. This equation[4] is a modification of the approximation equation used in water analysis for computing the Most Probable Number of coliform organisms present in a water sample.

[4] The equation, as modified by Chang and Baxter (1955), is as follows:

$$\text{MPN of surviving cysts per ml of test fluid} = \frac{(\text{Total No. pos. tubes}) \times (\text{No. cysts per ml test fluid})}{\sqrt[2]{(\text{Total No. cysts in neg. tubes}) \times (\text{Total No. cysts in all tubes in the series})}}$$

The irradiated suspension, containing about 5,000 cysts per ml, was adjusted by serial decimal dilutions to concentrations of 50, 5, and 0.5 cysts per ml. In the last case, the number 0.5 represents an even chance of withdrawing or not withdrawing a single cyst with a milliliter of fluid. One milliliter of each dilution was introduced onto each of five REL slants; these five tubes constituted a set. After incubating for 48 hours the sediments of the slants were inspected for the presence of trophozoites. Excystation and multiplication of amebae were accepted as evidence of the viability of at least some of the irradiated cysts. All slants were observed for at least 6 days before being discarded as negative.

A sample set of data with its MPN calculation was obtained in the following way. The data represent the controls from an irradiation experiment:

No. cysts/ml	Number positive tubes out of five
50	5
5	2
0.5	0

Omitting from the calculation the tubes with the largest inoculum which were all positive, the total number of positive tubes was two. The suspension originally contained 5,000 cysts per ml. There were eight negative tubes, three of which, as estimated, received five cysts each and five of which received 0.5 cyst each; or, a total of 17.5 cysts in the negative tubes. There were 27.5 cysts in the computed tubes in the series. Substituting these values in the MPN equation gives:

$$\text{MPN} = \frac{(2)(5{,}000)}{\sqrt[2]{(17.5)(27.5)}} = 450.0$$

This number is interpreted to mean that of 5000 cysts, 450 most probably were capable of excystation. Or the rate of viability was 9%.

Experimental

Irradiation of NRS Cysts

Cysts were exposed to gamma rays for varying time intervals at a distance from the source at which ionization was occurring at an intensity of about 840 RAD per minute. The writer was counseled not to attempt experiments employing exposures of less than 10 minutes (i.e., 8,400 RAD) since validation of the dose for shorter periods would be difficult. Exposure times were 10, 15, 18, 20, 25, 30, and 60 minutes. These intervals corresponded to dosages ranging from 8,400 to 50,200 RAD. Control suspensions in each experiment were placed in the labyrinthine entrance to the radiation cave where gamma radiation was negligible.

MPN values were calculated for control suspensions and irradiated suspensions, using as a value for the "total number of cysts in the suspension" the visualized count obtained from the hemocytometer. The ratio of the MPN of survivors in the irradiated group to the MPN of survivors in its respective control group was then determined and expressed as a *percentage of survival following irradiation*. A list of these data is presented in Table I, and the values are plotted in Fig. 1, where values on the vertical axis represent percentages of survival of irradiated cysts relative to the survival of unirradiated controls. The scarcity of points does not warrant attempting to fit a line. Nevertheless, a linear function is strongly suggested by the distribution of points. This does not contradict the hypothesis that the destruction of cysts by gamma rays is, like that of trophozoites, an exponential function of dosage, the reaction being of the first order. Killing of all viable cysts was always

TABLE I

Rate of Viability of Irradiated Cysts

Test No.[a]	Time in minutes	Dosage in RAD	Percentage of survival[b]
1	10	8,400	20.0
2	10	8,400	14.2
3	10	8,400	11.7
4	10	8,400	9.8
5	10	8,400	5.7
6	15	12,300	19.8
7	15	12,300	12.4
8	15	12,300	6.5
9	15	12,300	6.2
10	15	12,300	5.4
11	15	12,300	3.6
12	15	12,300	0.2
13	18	15,100	2.1
14	18	15,100	2.0
15	20	16,700	1.1
16	20	16,700	0.22
17	25	20,900	0.17
18	25	20,900	0.06
19	28	23,400	0.14
20	30	25,100	0.0
21	30	25,100	0.0
22	30	25,100	0.0
23	30	25,100	0.0
24	30	25,100	0.0
25	60	50,200	0.0
26	60	50,200	0.0

[a] The values for each dosage have been arrayed to show progressively smaller percentages of survival. Tests are *not* presented in the order performed.

[b] This value represents the ratio: MPN of survivors in irradiated group/MPN of survivors in control group.

achieved at doses of 50,200 and 25,100 RAD. Results of exposures at 23,400 and 20,900 RAD suggest that this order of magnitude of radiation insult represents the upper limit of cyst resistance. For technical reasons no tests were performed with exposures of less than 8,400 RAD (see above).

Irradiation of Cysts of Different Ages

To obtain the routine destruction of all trophozoites in the cyst suspensions, these

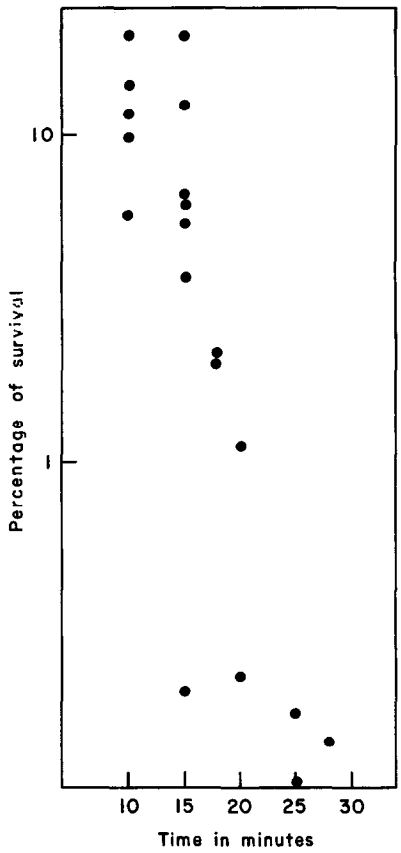

FIG. 1. Rate of viability of irradiated cysts.

were treated with distilled water and stored at 4–6°C overnight. The cysts were always used the following day. It seemed possible that "aging" of the cysts might constitute a variable factor capable of altering the radiation response. In order to acquire evidence to support this suspicion, an experiment was planned in which cysts were irradiated on the day of harvest as well as on the following day after a night in the cold. Thus, at the time of irradiation the respective ages of these cysts were 4 and 28 hours (counting from the time of harvest). The former will be termed "young" and the latter "old" cysts. Unirradiated controls were carried along with each experiment, as usual.

Table II presents quantitative information deriving from three sets of experiments, using cysts of different ages, in which doses of 8,400, 12,100, and 14,900 RAD were employed. Despite inconsiderable differences in

TABLE II

Comparison of the Results of Irradiation of "Young" and "Old" Cysts

Experiment	Age	Dose in RAD	Inoculum (cysts/ml)	MPN survivors	Percentage of survival
1	Young-Control	—	4,900	202	
	Young Irrad.	8,400	4,900	20	9.8
	Old-Control	—	4,900	139	
	Old Irrad.	8,400	4,900	8	5.7
2	Young-Control	—	6,600	407	
	Young Irrad.	12,600	6,600	8	1.98
	Old-Control	—	7,500	456	
	Old Irrad.	12,600	7,500	28.6	6.2
3	Young-Control	—	5,900	4023	
	Young Irrad.	15,100	5,900	81	2.01
	Old-Control	—	6,400	1376	
	Old Irrad.	15,100	6,400	28.6	2.07

concentration of cysts, time for preconditioning growth tubes, and temperature in the radiation cave, it was evident that the main difference between the suspensions in the "young" and "old" groups was the age of the cysts.

In Experiment 3 (Table II), the values for percentage of survival in both groups are seen to resemble each other closely. In Experiments 1 and 2, the differences of, respectively, 4.1% and 4.2% between young and old groups may be meaningful; however, differences this large have been observed with identical dosages when the cyst "ages" were the same. Besides, in Experiment 2, the greater percentage of survival occurs in the "old" group, whereas in Experiment 1 it is the "young" group which seems to be less radiosensitive.

It was concluded, then, that these data do not markedly support a hypothesis that a difference of 24 hours in the age of cysts can influence their response to gamma rays.

TABLE III

Comparison of Growth of Irradiated Cysts Alone and Irradiated Cyst-Trophozoite Mixture

Type of suspension	Dose in RAD	Time in minutes	Number of organisms per ml in test susp.	MPN of survivors	Percentage of survival
Cysts only	12,300	15	5,800	81	5.9
Control	—	—	5,800	1397	
Cysts & trophs	12,300	15	46,000	3018	74.5
Control	—	—	46,000	4047	
Cysts & trophs	124,200	55	46,000	20	0.49
Control	—	—	46,000	4047	

Radiosensitivity of Cysts and Trophozoites of the Same Strain

The data from these experiments have indicated that the cysts of *E. histolytica* were much more sensitive to gamma rays than the trophozoites. However, the work with trophozoites (Schneider and Porter, 1960) was performed with a different strain and at a different time. Thus, it seemed advisable to compare the reactions of cysts and trophozoites of the same strain by irradiating them together.

A pure cyst suspension containing 5,800 cysts per milliliter was prepared in the usual manner. However, a pure suspension of trophozoites could not be so easily obtained since some cysts were always present during the normal course of *in vitro* growth. Thus, by harvesting NRS amebae at 48 hours of growth, a suspension was obtained in which approximately 70% of the organisms were trophozoites. This preparation contained 46,000 trophozoites per milliliter.

The trophozoite suspension was divided into two portions. One of these was exposed to 29,800 RAD, a dose identical with that used on the cysts. The second portion was exposed to the same dose; it was then placed in the center well of the source for an additional forty minutes, thus increasing the overall dose to 124,200 RAD.

The results, presented in Table III, reveal that the suspension of cysts alone presented a degree of sensitivity in close agreement with the results of previous experiments. Reducing the experimental MPN to a per-

centage of the control MPN gave a figure of 5.9% survival of cysts at 29,800 RAD.

In the cyst-trophozoite mixture which received the same dose as did cysts alone, the ratio of test MPN to control MPN was far greater than that for cysts alone.

Finally, the cyst-trophozoite suspension which received more than 124,200 RAD showed a low survival ratio (Table III). Such a level of radiation is almost ten times that which has always proved completely lethal for cysts alone. It is felt that this is a significant difference, considering the order of magnitude. However, no firm claim can be made for the perfect accuracy of the dosage values; these remain, as always, approximations.

Discussion

One plausible interpretation of the difference between cyst and trophozoite sensitivity postulates the existence of certain radiation-susceptible structures (possibly thiol groups), the minimum critical number of which may be the same for both trophozoite and cyst but which the trophozoite may originally possess in greater abundance than the cyst. If true, the level of ionizing insult required to inactivate the organism permanently would have to be greater for the trophozoite (to bring the number of such structures below the hypothetically vital minimum) than for the cyst.

An alternative explanation involves the fact that the permeability of the cyst wall is much lower than that of the trophic pellicle. It is likely that injurious substances are formed within the organism during irradiation. Assuming that the active trophozoite can alter or expel these substances with more facility than can the dense-walled cyst, there would remain only to identify the poisons formed and account chemically for their presence. Hypothetically, hydrogen peroxide would serve very well as such a substance; it is formed from thiol compounds during irradiation (Barron and Flood, 1950). Moreover, there is presumably no catalase in the anaerobic amebae to help them combat the intoxication.

A fully satisfactory explanation of this problem can be advanced only after further experimental work has been done.

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