STUDIES ON SUSCEPTIBILITY TO INFECTION FOLLOWING IONIZING RADIATION

V. Comparison of Intraperitoneal and Intravenous Challenge at Intervals Following Different Doses of X-radiation*

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Enhanced susceptibility to bacterial infection is one of the important changes which follow whole body exposure of mammals to moderate doses of ionizing radiation. Its occurrence during the post-irradiation period has been well documented by laboratory and clinical observation, but the time of its onset and duration has not been studied as systematically as it deserves.

Schechmeister, Bond, and Swift (1) infected mice by inhalation of an aerosol of *Streptococcus zooepidemicus* at intervals after total body exposure to 350 r x-radiation. The ratios of the LD_{50}'s of normal mice to those of irradiated mice plotted against time showed that susceptibility to this air-borne infection increased steadily until the 15th day post-irradiation, then declined at the same rate to the 30th day, and slowly returned to normal by the 41st day.

Kaplan, Speck, and Jawetz (2) related susceptibility to time post-irradiation (450 r) by plotting average day of death after intramuscular injection of a standard inoculum (100 LD_{50}) of a beta hemolytic streptococcus (strain C203). By these criteria, susceptibility of the mice was maximal between the 3rd and 7th day after x-radiation. However, Schechmeister, Paulissen, and Fishman (3) reported that they found susceptibility of mice to infection with *Salmonella enteritidis* inoculated intraperitoneally to be maximal 8 hours after irradiation with 350 r; but survival times were not reported, and it is well known that *Salmonella enteritidis* infection may not kill for a number of days after inoculation.

In a small series of experiments, Marston *et al.* (4) found that susceptibility did not continue to increase beyond the 3rd day post-irradiation (475 r) among mice injected subcutaneously with a standard inoculum of a virulent strain of *Proteus vulgaris* (Kf7).

Hammond, Colling, Cooper, and Miller (5) inoculated RAP female mice 9 to 10 weeks old, with approximately 10^7 *Pseudomonas aeruginosa* by stomach tube at different times (2 hours, 5 days, and 11 days) after whole body exposure to 550 r. Mor-

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tality from *Pseudomonas* bacteriemia was greatest among the mice inoculated on the 11th day post-irradiation and least among those inoculated 2 hours after irradiation.

In the experiments herein described, young adult mice were exposed to one of the following doses of x-radiation—300, 400, 475,[1] 500, and 600 r—in order to determine the effect of each dose on susceptibility to an experimental bacterial infection during the first 3 weeks post-irradiation. This is a period when many pathological changes are known to occur in rapid succession. It was necessary, therefore, to make frequent determinations and to assay susceptibility by inducing an acute, rapidly fatal infection so that the lapse of time between inoculation and death should be as short as possible. ***Pseudomonas aeruginosa*** was used because it kills more quickly than any microorganism we have tried for this purpose.

Materials and Methods

Mice.—CF-1 females, 10 weeks old, weighing 20 to 25 gm. were used. They were 8 weeks old on arrival and were held in quarantine and examined daily for 2 weeks. If any evidence of *Salmonella* or other epizootic infection appeared, the whole shipment was discarded. All the mice challenged at one time came from a single shipment. They were housed on bedding of wood shavings, in groups of 10 to 20, in stainless steel cages measuring 15 x 9 x 7 inches. Cages were changed and autoclaved twice a week. Rockland mouse pellets were available at all times as was tap water in sterilized water bottles which were changed daily.

Irradiation.—The mice were irradiated with a 250 kv., 30 ma. maxitron 250 (General Electric) machine using 0.25 mm. copper and 1 mm. aluminum filters at a target distance of 70 cm. and a dose rate of approximately 60 r per minute.[2] For details of the method of exposure see Hammond, Ruml, Cooper, Miller. (6)

The largest series of experiments was made with mice exposed to 500 r for two reasons: (*a*) it was a dose which was usually sublethal, *e.g.*, it killed only 3 among 100 uninoculated controls in 8 runs; and (*b*) early experiments had showed this dose to produce a marked effect on susceptibility to infection. Lower and higher doses were used in smaller series of experiments—300, 400, 475, and 600 r. The results in this last series were not wholly comparable with the others because 600 r was an LD_{50-70} for these mice.

Challenge Microorganism.—This was a streptomycin-resistant strain of *Pseudomonas aeruginosa* which has been used for challenge in this laboratory for a number of years. Its virulence for unirradiated CF-1 mice, maintained by weekly passage, has been remarkably constant. Its streptomycin resistance served merely to differentiate it in autopsy cultures from any other strains of *Pseudomonas aeruginosa* which the mice might have been carrying in their intestinal tracts.

An 18 hour culture on an agar plate was suspended in 5 ml. saline, and put into a 25 ml. Erlenmeyer flask containing a few glass beads and agitated on an electric rotator for 15 minutes to disperse any clumps of bacteria. The suspension was then diluted to a standard density (approximately 10^9 microorganisms per ml.) by means of a Coleman spectrophotometer. Tenfold dilutions were made and the bacterial content checked by plating, in quadruplicate, 0.1 ml. of the 10^{-6} and 10^{-7} dilutions.

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[1] A single experiment at 475 r.

[2] Irradiation was carried out at the Argonne Cancer Research Hospital, Chicago, operated by the University of Chicago for the United States Atomic Energy Commission.

The same suspensions were used for intravenous and intraperitoneal inoculations. Plate counts made after completion of the inoculations showed that practically no change occurred in the numbers of viable microorganisms in the suspensions during the several hours required to inject all the mice in the larger experiments.

The challenge inoculations were planned so that the largest inocula would kill all of the mice and the smallest none.

Inoculation.—The mice were randomly distributed into groups of 10 to 20 and were then injected intraperitoneally or intravenously (into the dorsal tail vein) with 0.5 ml. quantities of appropriate dilutions, using a 27 gauge needle. This volume was used because it could be measured more accurately than a smaller one and was found to be not too large for intravenous inoculation. Great care was taken with all intravenous injections and any mouse was discarded if the needle failed to enter the vein on the first attempt or if any leakage of inoculum into the perivenous tissue was detected. Survivors were observed for 30 days after inoculation.

Both intraperitoneal and intravenous routes of inoculation were used in the 400 and 500 r series, but only the intraperitoneal in the 300 and 600 r series.

Unirradiated controls were not included in every challenge inoculation because their LD_{50}'s were in such close agreement. When they were included, they came from the same shipment as the irradiated mice.

Methods of Calculating LD_{50}.—Both least squares and probit analysis were used to calculate the LD_{50}'s. The values obtained by the two methods were in very close agreement, varying not more than 0.3 of a log. The LD_{50}'s listed in Tables I and II and the points plotted in Figs. 1, 2, and 4 were those obtained by the method of least squares.

Autopsies.—Every mouse which died was autopsied for culture of heart's blood unless it had been eaten by its cage mates. A few mice which died with negative blood cultures or with blood cultures positive for some other microorganism were excluded from the mortality data.

Leucocyte Counts.—On the days indicated in Fig. 3 and Table III, white blood counts were made on uninoculated mice in each series. Blood was drawn from a dorsal tail vein and the counts were made by the customary methods.[3] No mouse was used more than once for this purpose. Differential counts were also made on representative samplings of mice.

RESULTS

400 and 500 r Series.—Both intravenous and intraperitoneal routes of inoculation were used in these series. Tables I and II list: (*a*) the LD_{50} of the inocula in each challenge, (*b*) the average of these for each day of challenge (post-irradiation interval), and (*c*) the LD_{50} computed from the pooled mortality data for each day of challenge. It is these last computations (LD_{50} of the pooled mortalities) which are plotted in Figs. 1 and 2. Table II which presents the data on the 500 r series also includes the results, in parentheses, of a single experiment on mice irradiated with 475 r.

In both of these series, susceptibility to the experimental infection increased rapidly to the 3rd day post-irradiation. In the 400 r series, it declined thereafter; but in the 500 r series, it was maintained until the 11th day. In both series susceptibility had returned to normal by the 20th day post-irradiation.

300 and 600 r Series.—These mice were challenged at only 3 intervals after

[3] The leucocyte counts were made by Frances S. Vandervoort, Betty Wolfe, and Gilbert Claudio.

TABLE I

Results of Challenge Inoculations—400 r Series

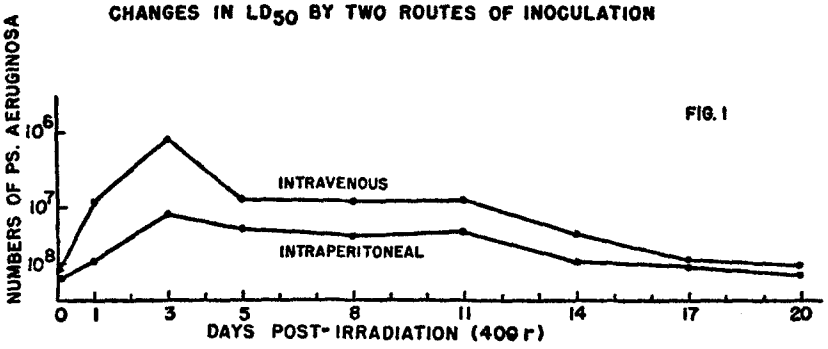
Day post-irradiation	Intravenous				Intraperitoneal			
	LD_{50}	No. mice	Averaged LD_{50}'s	LD_{50} of pooled mortality data	LD_{50}	No. mice	Averaged LD_{50}'s	LD_{50} of pooled mortality data
1	4.8×10^6	40	9.4×10^6	9.4×10^6	4.7×10^7	40	7.3×10^7	7.0×10^7
	1.4×10^7	60			1.0×10^8	67		
3	1.0×10^6	80	2.1×10^6	1.5×10^6	8.7×10^6	96	1.2×10^7	1.4×10^7
	3.2×10^6	96			2.6×10^7	72		
5	3.0×10^6	40	8.2×10^6	8.1×10^6	1.4×10^7	40	2.4×10^7	2.0×10^7
	6.6×10^6	40			2.0×10^7	40		
	1.5×10^7	80			4.0×10^7	80		
8	3.2×10^6	80	1.0×10^7	9.5×10^6	2.8×10^7	80	2.9×10^7	3.0×10^7
	1.8×10^7	80			3.0×10^7	80		
11	6.8×10^6	80	8.4×10^6	8.2×10^6	9.5×10^6	80	2.5×10^7	2.4×10^7
	1.0×10^7	95			4.0×10^7	76		
14	3.1×10^7	80	3.1×10^7	3.1×10^7	4.8×10^7	80	5.0×10^7	5.1×10^7
	3.2×10^7	80			5.2×10^7	80		
17	4.0×10^7	80	5.8×10^7	5.4×10^7	6.8×10^7	60	7.6×10^7	7.3×10^7
	7.6×10^7	76			8.4×10^7	57		
20	6.2×10^7	80	7.2×10^7	7.3×10^7	6.4×10^7	60	7.7×10^7	8.0×10^7
	8.3×10^7	54			9.1×10^7	54		
Normal Controls	7.1×10^7	30	7.5×10^7	7.5×10^7	7.4×10^7	30	7.9×10^7	8.1×10^7
	7.3×10^7	30			7.6×10^7	30		
	7.5×10^7	24			8.0×10^7	24		
	7.9×10^7	30			8.2×10^7	30		
	8.0×10^7	30			8.3×10^7	30		

irradiation and only by intraperitoneal inoculation. Exposure to 300 r caused but slight increase in susceptibility to the experimental infection. Exposure to 600 r, however, was followed by a very marked increase. This result was not unexpected because this dose of radiation is an $LD_{50\text{-}70}$ for these mice (Fig. 4).

Duration of the Lethal Infections in Irradiated Mice.—As was pointed out in the introduction, *Pseudomonas aeruginosa* was chosen as the test microorganism because it produces a rapidly lethal infection. Deaths among unirradiated mice usually occurred within 18 to 30 hours, occasionally as late as 48 hours, only rarely thereafter. Although most deaths among irradiated mice followed this

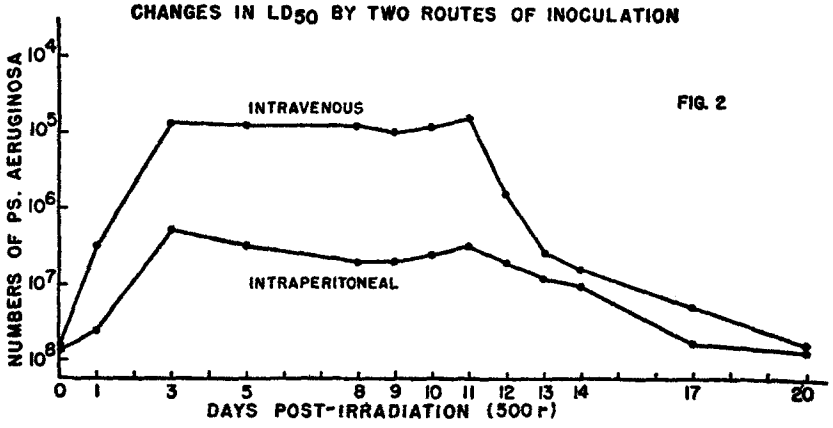
CHANGES IN LD_{50} BY TWO ROUTES OF INOCULATION

FIG. 1



CHANGES IN LD_{50} BY TWO ROUTES OF INOCULATION

FIG. 2



GEOMETRIC MEANS OF LEUKOCYTE COUNTS OF MICE IRRADIATED WITH 400 AND 500 r

FIG. 3

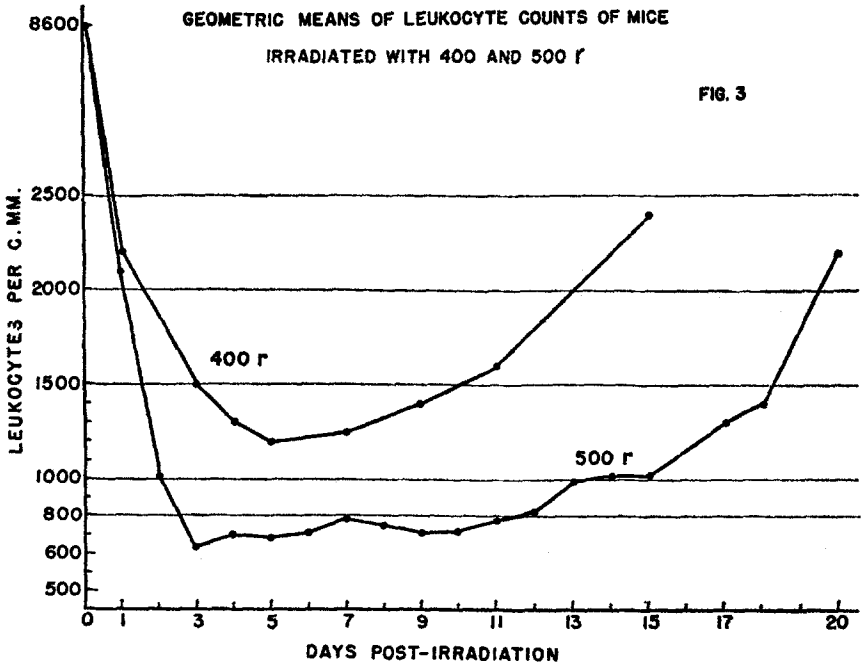


TABLE II

Results of Challenge Inoculations—500 r Series (and One Experiment with 475 r)*

Day post-irradiation	Intravenous				Intraperitoneal			
	LD_{50}	No. mice	Averaged LD_{50}'s	LD_{50} of pooled mortality data	LD_{50}	No. mice	Averaged LD_{50}'s	LD_{50} of pooled mortality data
1	1.0×10^6	30	3.1×10^6	3.2×10^6	2.6×10^7	30	3.8×10^7	4.3×10^7
	5.2×10^6	56			5.0×10^7	50		
3	8.7×10^4	50	1.4×10^5	9.2×10^4	1.0×10^6	40	2.6×10^6	2.3×10^6
	2.0×10^5	50			4.2×10^6	40		
5	3.0×10^4	30	1.6×10^5	9.4×10^4	1.0×10^6	50	3.9×10^6	3.4×10^6
	2.3×10^5	50			3.4×10^6	50		
	2.7×10^5	78			7.4×10^6	65		
8	6.2×10^4	55	9.4×10^4	9.5×10^4	4.3×10^6	44	5.8×10^6	5.8×10^6
	7.1×10^4	120			6.2×10^6	80		
	1.5×10^5	78			7.1×10^6	78		
9	1.6×10^4	78	9.1×10^4	1.3×10^5	9.8×10^5	80	5.6×10^6	5.9×10^6
	5.8×10^4	114			5.8×10^6	78		
	2.0×10^5	80			1.0×10^7	120		
	(4.1×10^5)*	80			(6.5×10^7)*	114		
10	6.1×10^4	65	7.4×10^4	7.6×10^4	9.7×10^5	77	3.2×10^6	4.5×10^6
	6.3×10^4	133			1.0×10^6	110		
	7.2×10^4	40			2.3×10^6	40		
	1.0×10^5	40			8.8×10^6	40		
11	6.6×10^3	60	7.0×10^4	5.2×10^4	8.3×10^5	30	2.9×10^6	3.2×10^6
	3.5×10^4	50			1.0×10^6	50		
	5.2×10^4	50			2.5×10^6	50		
	7.7×10^4	36			3.3×10^6	60		
	1.8×10^5	60			7.1×10^6	80		
	(2.3×10^6)*	80			(3.4×10^6)*	60		
12	5.9×10^5	72	1.3×10^6	7.0×10^5	6.7×10^6	72	6.9×10^6	6.2×10^6
	2.1×10^6	84			7.1×10^6	60		
13	1.0×10^6	94	7.0×10^6	4.4×10^6	8.7×10^6	65	8.8×10^6	8.9×10^6
	1.3×10^7	65			9.0×10^6	61		
	(4.2×10^5)*	80			(1.1×10^7)*	80		
14	2.0×10^6	40	6.5×10^6	7.1×10^6	6.6×10^6	40	1.4×10^7	1.0×10^7
	1.1×10^7	72			2.3×10^7	72		
17	1.6×10^7	65	1.6×10^7	2.4×10^7	5.0×10^7	60	6.1×10^7	6.8×10^7
	1.6×10^7	75			7.2×10^7	40		
20	7.2×10^7	54	7.2×10^7	7.2×10^7	7.4×10^7	42	7.4×10^7	7.4×10^7

* Results of one experiment with 475 r are shown in parentheses, but not included in averaged or pooled LD_{50}'s.

TABLE II—*Concluded*

	Intravenous				Intraperitoneal			
	LD_{50}	No. mice	Averaged LD_{50}'s	LD_{50} of pooled mortality data	LD_{50}	No. mice	Averaged LD_{50}'s	LD_{50} of pooled mortality data
Normal Controls	7.3×10^7	30	7.6×10^7	7.7×10^7	7.4×10^7	30	8.0×10^7	8.1×10^7
	7.4×10^7	30			7.5×10^7	30		
	7.5×10^7	24			7.7×10^7	30		
	7.7×10^7	30			8.0×10^7	30		
	8.0×10^7	24			8.4×10^7	30		
	8.1×10^7	30			8.5×10^7	30		

TABLE III

Total Leucocyte Counts

Days post-irradiation	300 r	600 r
1	3000	2100
5	2400	500
11	4200	750

TABLE IV

Differential Leucocytes Counts

Lymphocytes (L) and Polymorphonuclear Heterophiles (P) Per Cent

Averages of 10–15 Mice

Day post-irradiation	400 r		500 r		600 r	
	L	P	L	P	L	P
1	66	34	58	42	66	34
5	70	30	79	21	92	8
11	80	20	99	1	99	1

Unirradiated controls: lymphocytes, 70 per cent; polymorphonuclear heterophiles, 25 per cent.

time schedule, some were delayed beyond it. With few exceptions these late deaths resulted from intravenous inoculation of the lower doses of *Pseudomonas aeruginosa*. As they were shown by positive heart's blood culture to have been due to *Pseudomonas* infection, and as they constituted only a small fraction of the total, they were included in the mortality data.

Leucocyte Counts.—The geometric means of the leucocyte counts on 120 uninoculated mice exposed to 400 r and on 596 exposed to 500 r are plotted in Fig. 3. In the 400 r series, the lowest level (1200) occurred on the 5th day

post-irradiation, after which the counts rose steadily. In the 500 r series, they fell much more steeply to a minimum of 640 on the 3rd day and remained below 800 until the 12th day.

The geometric means of the leucocyte counts on 90 uninoculated mice exposed to 300 r and 111 exposed to 600 r are shown in Table III.

Differential leucocyte counts are presented in Table IV as percentages of lymphocytes and polymorphonuclear heterophiles. The other types of leucocytes have been omitted from the table, as they were never numerous and be-

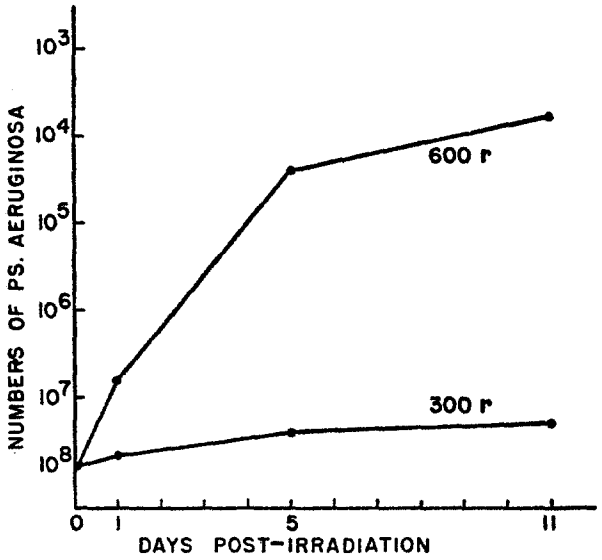


FIG. 4. Changes in LD_{50} following 300 and 600 r.

came very scarce during the periods of leucopenia. The most interesting finding was the almost total disappearance of polymorphonuclears on the 11th day in the 500 and 600 r series.

Comparison of Results of Challenge by Intravenous and Intraperitoneal Routes of Inoculation.—According to the curves in Figs. 1 and 2 and the data in Tables I and II, irradiated mice were much more susceptible to the experimental infection when it was initiated by intravenous inoculation. This difference was due to the fact that equivalent numbers of *Pseudomonas aeruginosa* caused more deaths when they were injected intravenously than when they were injected intraperitoneally. Among unirradiated mice, however, there was practically no difference in the mortalities resulting from the two routes of inoculation.

In search of an explanation for this disparity, additional experiments were carried out. The results, summarized below, show the greater lethality of intravenously induced infection in irradiated mice to have been due to the

establishment of a focus of infection resulting from leakage of a minute fraction of inoculum into the perivenous tissues at the site of injection.

(*a*) Leakage into the perivascular tissues during intravenous inoculation was demonstrated by injecting India ink into the tail vein with the same care as was used in the inoculations. After amputation, dehydration, and clearing, a small spot of India ink was distinctly visible by transmitted light at the site of the injection.

(*b*) Microscopic studies were made of sections of the tails of mice inoculated intravenously with staphylococci, which could be identified in stained prepara-

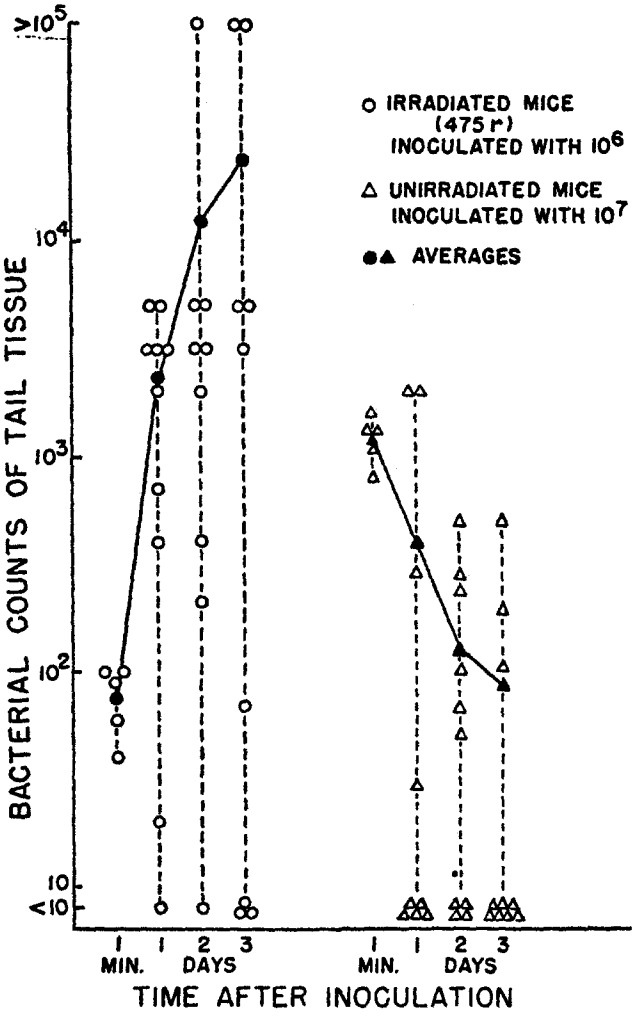


FIG. 5. Numbers of *Ps. aeruginosa* recovered from homogenates of tissue at site of intravenous inoculation.

tions more accurately than *Pseudomonas*. As late as 48 hours after injection, staphylococci were to be seen in and around the inoculated vein. In unirradiated mice the perivenous tissue was infiltrated with leucocytes, while in irradiated mice (5 days after 500 r) almost no leucocytes were present and the staphylococci were much more numerous; *i.e.*, had multiplied in the irradiated mice.

(*c*) Normal and irradiated mice (on the 5th day after 500 r) were inoculated intravenously with *Pseudomonas aeruginosa*, the normals with 10^7 and the irradiated mice with a tenth that number (10^6), since the larger dose would have been lethal for them. One minute later and on the 3 succeeding days, mice were killed and the tissues about the site of injection were excised, homogenized in streptomycin broth, and cultured by plating 0.1 ml. of tenfold dilutions.

The results, plotted in Fig. 5 show that shortly after an intravenous injection, approximately 0.0001 of the bacteria in each inoculum was recovered from the subcutaneous tissues at the site of inoculation. The results also show that during the next 3 days, the average numbers of these bacteria increased in the irradiated mice but decreased in the unirradiated mice.

The irradiated (leucopenic) mice were unable to dispose of these extravascular bacteria which were able to multiply and create a focus of infection, a process which may have been facilitated by the toxin produced by *Pseudomonas aeruginosa*.

Such foci of infection were not found in the abdominal wall at the site of intraperitoneal inoculation even in irradiated mice.

DISCUSSION

In this investigation the effect of four levels of x-radiation on susceptibility to bacterial infection was studied by comparing a series of LD_{50}'s, determined at intervals post-irradiation, with those obtained on unirradiated mice. The LD_{50}'s were estimated by customary methods from mortality data on mice challenged with graded inocula of the test microorganism, *Pseudomonas aeruginosa*. Such a method of biological assay inevitably involves uncontrollable factors which are increased when it is applied to an animal host suffering from the radiation syndrome.

As is well known, exposure to doses above 300 r initiates a sequence of pathological changes which do not develop at the same rate or to the same degree in all members of a mouse population. In other words, innate or at least inherent differences in radiosensitivity seem to exist among individuals of the same age and origin. The mice used, of necessity, in these experiments were not a highly inbred strain. To compensate for their lack of uniformity, large numbers were used and the results checked by repetition.

Although our quantitative estimations of susceptibility to the bacterial infection (LD_{50}'s) must be regarded as approximations, the results do show certain definite effects: Intraperitoneal inoculations in the four series show, as

had been expected, that the larger the dose of radiation the greater the effect on susceptibility to the experimental infection. Exposure to 300 r caused practically no change; 400 r caused a moderate but transitory, and 500 r a marked and prolonged effect. 600 r produced the most marked one, but caused so many radiation deaths that the results of the inoculations are not comparable with the others.

In the 400 and 500 r series, in which two routes of inoculation were used, susceptibility to the experimental infection appeared to have been increased much more in the mice challenged by intravenous inoculation than in those challenged intraperitoneally.

Among irradiated mice higher mortalities resulted from equivalent inocula injected into the tail vein because a local infection was established at the site of injection. Their leucopenia prevented the irradiated mice from combating this infection which added substantial numbers of bacteria to those which had already been inoculated. The establishment of the local infection was probably facilitated by the toxin produced by the test microorganism. Such foci of infection were not found at the site of intraperitoneal injection, by which route the point of the needle pierces the abdominal wall very easily and rapidly, with a minimum of mechanical injury. Injection into a tail vein is a slower and more painstaking procedure and inevitably results in some degree of local trauma and leakage of a minute fraction of the inoculum into the surrounding tissues which have a poorer vascular supply than those of the abdominal wall.

Comparison of the curves in Figs. 1, 2, and 3 brings out the correlation of severity of leucopenia with increased susceptibility to infection. It is particularly striking in the case of intravenously inoculated mice in the 500 r series, presumably for the reasons described. Smith *et al.* have emphasized the importance of leucopenia, especially granulocytopenia, as a factor in post-irradiation susceptibility to bacterial infection (7, 8).

SUMMARY

Ten week old female CF-1 mice were subjected to a single total body exposure of x-radiation in one of the following doses: 300, 400, and 500 r. At intervals thereafter, susceptibility to bacterial infection was determined by intraperitoneal challenge with graded inocula of *Pseudomonas aeruginosa*. Mice exposed to 400 or 500 r were also challenged by intravenous inoculation.

The LD_{50} of the test microorganism in each challenge was estimated from the mortality data.

Exposure to 300 r caused little increase in susceptibility to this experimental infection. 400 r caused a moderate increase on the 3rd day post-irradiation with return to normal on the 17th. Leucocyte counts (geometric means) following this dose of radiation did not fall below 1200.

500 r caused a marked increase in susceptibility which lasted from the 3rd to 11th day, during which period the leucocyte counts were below 800. On the 20th day, susceptibility to infection was normal, although the geometric mean of leucocyte counts was only 2200.

Comparison of mortalities resulting from equivalent inocula introduced by the two routes (intravenous and intraperitoneal) showed no difference in unirradiated mice. However, among mice irradiated with 400 or 500 r, higher mortalities resulted from intravenous inoculation. The difference was found to be due to the establishment of a small focus of infection at the site of intravenous injection as a result of leakage of a minute fraction of inoculum into the perivenous tissues of the tail. Bacterial multiplication occurred in such foci in irradiated (leucopenic) mice, but not in unirradiated mice, nor at the site of intraperitoneal inoculation even in irradiated mice.

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