The effect of gamma irradiation on *Nematospiroides dubius*. Factors affecting the survival of worms in a primary infection in mice

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ABSTRACT

The infective larvae of *Nematospiroides dubius* were exposed to various levels (0 – 30 krad) of gamma irradiation by means of a Cobalt 60 source. Groups of mice were infected with these larvae and autopsied 5 weeks later for worm counts. It was found that male worms were more susceptible to irradiation than female worms. In both instances, however, the survival curve on a semi logarithmic plot was characterised by a shoulder at low doses and an exponential component at the higher levels of exposure. No male worms were recovered from mice infected with larvae given more than 12 krad but some female worms were capable of surviving 20 krad. The fecundity of female worms was reduced by 61% at 4 krad and totally ablated at 8 krad.

Further experiments demonstrated that the survival of irradiated *N. dubius in vivo* was related to the extent of the damage caused at the time of irradiation and was not dependant on additional host parameters. Thus neither the number of irradiated worms inoculated nor the sex of the host radically altered the sex ratio or proportion of the worms lost as a result of irradiating the larvae. Furthermore, treatment with cortisone or sublethal irradiation of the host did not increase the proportion of surviving worms. It was, therefore, concluded that a host immune response was not involved.

The disruptive properties of ionising radiation on biological systems have been used for many years to attenuate pathogenic organisms and tumours in the search for effective vaccines against the diseases initiated by these agents (Anderson and Warner, 1976). Furthermore the literature records numerous attempts to attenuate the infective stages of parasites so as to elicit protection in the host without causing the disease (IAEA, 1968). Foremost among this work rank the experiments of Jarrett *et al* (1959) which led to the successful vaccine against Husk, *(Dictyocaulus viviparus),* as also Miller's subsequent work on canine hookworm disease (Miller, 1978). Despite these successes, however, there is still little basic information on the effect of radiation on the biology of many nematode parasites in common laboratory use.

One species which has not been studied in this way is the murine trichostrongylid nematode *Nematospiroides dubius,* a parasite which like many medically important species gives rise to a chronic primary infection. Preliminary experiments have indicated that irradiated larvae of this species stimulate greater immunity than normal worms (Behnke and Parish, in preparation) and therefore, before a thorough study of the factors involved in the induction of acquired immunity by irradiated larvae could be undertaken, it was necessary to seek baseline data about the effects of radiation on infective larvae in a primary infection. The present paper reports the results of a series of experiments in which we examined the effect of a number of different parameters on the course of primary infection with irradiated *N. dubius* in mice.

MATERIALS AND METHODS

Inbred male NIH mice were used throughout this work, unless otherwise stated. The mice were bred and maintained under conventional animal house conditions in the Zoology Department of Nottingham University and were used for experiments when

6 – 12 weeks old. The background and maintenance of the parasite, and the methods used for infection of animals, have been previously described (Behnke and Wakelin 1977). Worms were recovered from the intestines of mice by means of a modified Baerman technique. The mice were killed with chloroform and the entire small intestine was removed, carefully slit open and placed on a layer of silk gauze which was suspended in a 50 ml beaker filled with Hanks' saline. The beakers were incubated at 37°C for a minimum of 6 hours. After the completion of incubation 1 ml of formalin was added to each flask and these were stored at room temperature until examination was possible. The worms were transferred to a petri-dish and counted under a binocular dissecting microscope. This technique recovered 95 – 100% of worms from the intestinal lumen but did not recover worms which were still retained in the mucosa.

Faecal egg counts were carried out on one gram samples of pooled faeces from all the mice in each group, deposited over the preceding 24 hours. The faecal samples were dispersed in 8 ml of 50% saturated saline and washed through a sieve (aperture size 800 μm) with 35% zinc sulphate solution. The eggs were counted after flotation in standard McMaster counting slides and the counts were expressed as the number of eggs per gram of whole faeces. The relative fecundity (modified from Dineen and Wagland 1966) was calculated as the mean of the egg counts recorded between day 11 and the last day of the experiment.

The infective, third stage larvae of *N. dubius* were exposed to gamma radiation from a Cobalt 60 source in the Chemistry Department of Nottingham University. At least 50 ml of a larval suspension were prepared and adjusted to contain the required dose of larvae in 0.2 ml of suspension. Twenty ml aliquots were then transferred to plastic universal tubes, which were placed 10 cm from the source. At this distance the rate of exposure was approximately 1 krad per minute. The source was accurately calibrated by a simplified form of the Fricke method (Allen 1961) and the exposure time for precise doses was calculated with reference to Cobalt 60 decay tables. The temperature in the exposure chamber never exceeded 18°C and therefore the decrease in radio-sensitivity which is known to occur at higher temperatures should not have been apparent in our experiments (Fitzpatrick and Mulligan, 1968). After irradiation the contents of the universal tubes were transferred to 25 ml conical flasks and a magnetic stirrer was used to ensure a uniform dispersal of larvae in the inoculum.

Cortisone acetate (Cortistab, Boots) was given by subcutaneous injection every second day. The first two injections were 1.25 mg/mouse and the remainder at 0.625 mg/mouse. All the control and treated mice were given oxytetracycline hydrochloride (Terramycin, Pfizer Ltd) in their drinking water at a concentration of 3g/litre.

The results were analyzed for significance by the nonparametric Wilcoxon test (Sokal and Rohlf, 1969). A value of $P<0.05$ was considered to be significant.

RESULTS

Three experiments (Experiments 1 – 3) were carried out in which mice were infected either with 100 normal infective larvae or with 100 irradiated larvae of *N. dubius*. The time-course of infection was then followed by killing for worm counts, 3 – 4 animals from each group on days 10, 14, 21 or 35. The worm recoveries from experimental groups were expressed as a percentage of the control value and the results are summarised in Fig. 1.

There was no significant reduction in the number of worms recovered from groups given larvae irradiated at 2.5 or 5 krad and the worm burden in these groups remained relatively unaltered over the 5 week period of infection. However, irradiation at 10 krad or more resulted in a significant reduction in worm numbers and at the higher levels of irradiation (i.e. 15 krad and 20 krad) there was an additional gradual but consistent loss
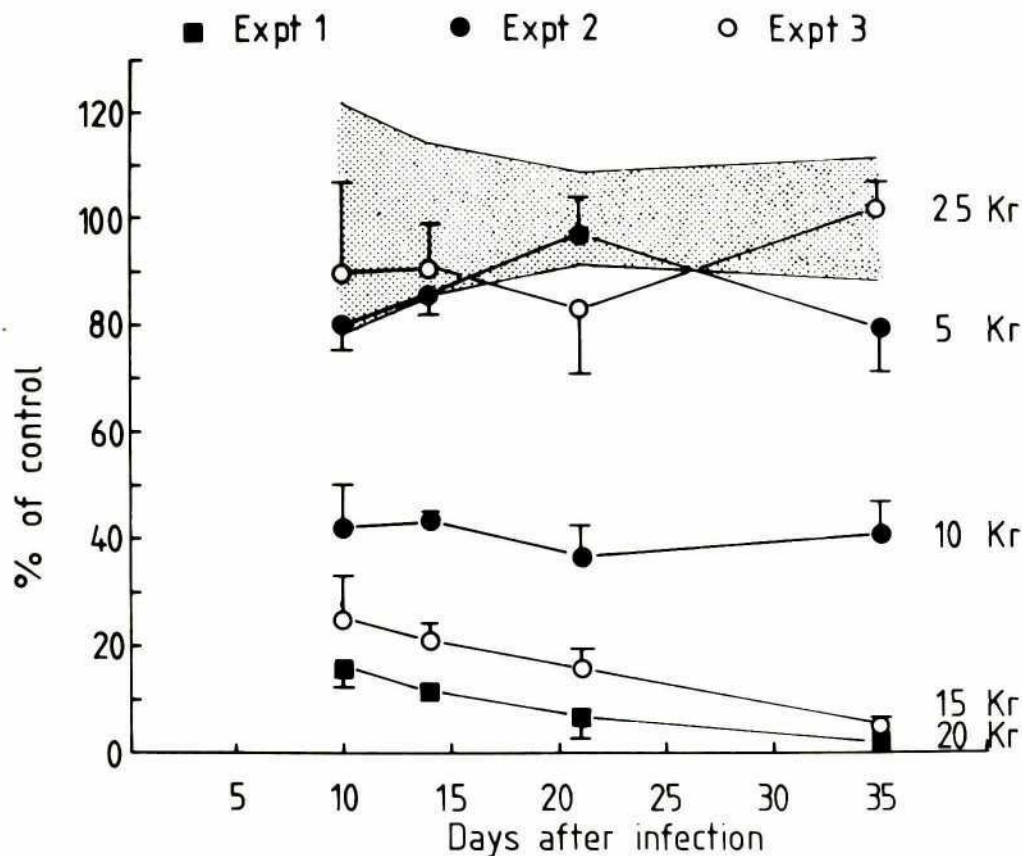
FIG. 1. Experiments 1 – 3. The effect of different levels of irradiation on the time course of infection. Three separate experiments were carried out (illustrated by the different symbols) in which the mice were infected with 100 control or irradiated larvae of *N. dubius* and were killed for worm counts in groups of 3 – 4 on the days shown. The results are expressed in terms of mean percentage (± S.D.) of worms recovered from control mice (One S.D. is illustrated for each point on the Figure). The stippled area represents the variation (S.D.) in worm recovery from the control groups, in which the overall mean worm burden was 83.0 ± 11.0, 91.7 ± 10.9 and 74.3 ± 6.4 respectively (Kr = krad).

of worms throughout the experiment. It is evident from these results that a proportion of worms was severely impaired and thus not recovered after irradiation at levels exceeding 5 krad and that the proportion of worms affected was related to the dose of irradiation administered.

During experiments 1 – 3 it was noted that male worms appeared to be more susceptible to irradiation than female worms. In order to clarify this point and to determine the precise relationship between the dose of irradiation administered and the percentage of worms surviving to day 35, three additional experiments were carried out (Experiments 4 – 6). Groups of six mice were infected either with normal larvae or with irradiated larvae and were killed for worm counts on day 35. Male and female worms were counted separately and the results for experimental groups were expressed as a percentage of the control value. Figure 2 shows the pooled results of these experiments, together with additional data from Experiments 7, 8 and 9.
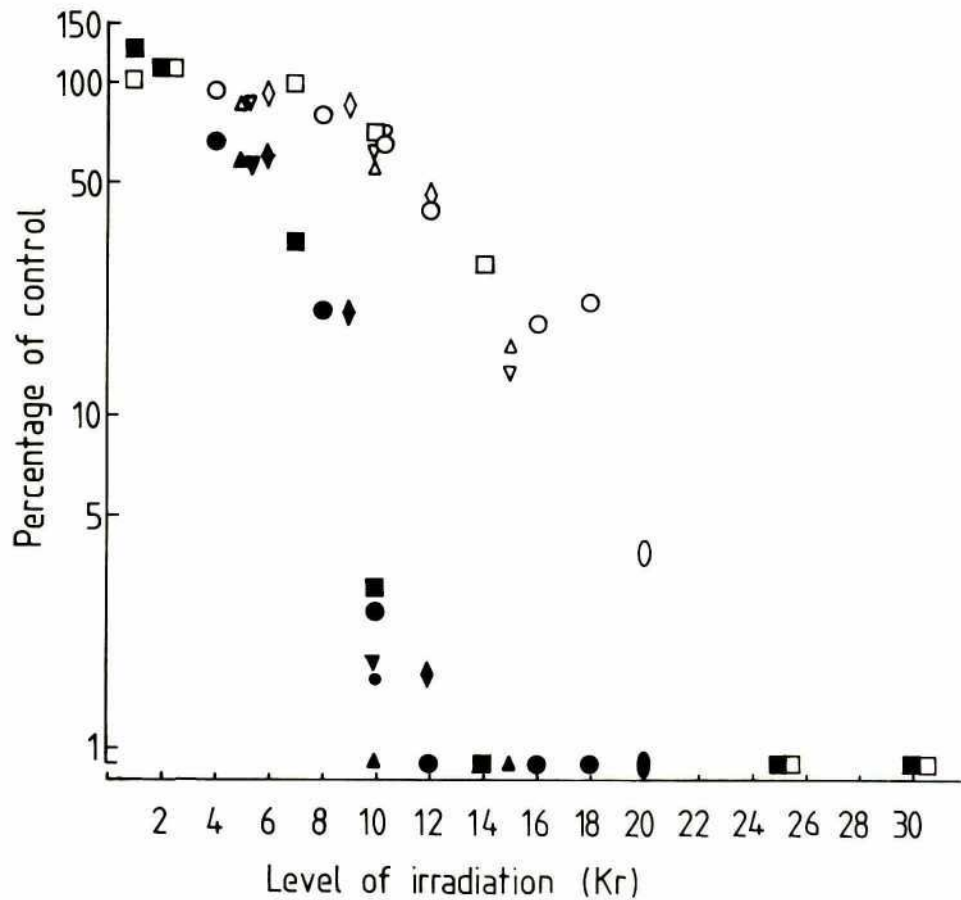
FIG. 2. Experiments 1 and 4 – 9. The relationship between the dose of irradiation administered to the larvae and the number of male and female worms surviving until day 35. Groups of 4 – 6 mice were infected with 100 or 120 (Experiment 7) control or irradiated larvae of *N. dubius* and were killed for worm counts on day 35. The results are expressed in terms of precentage of worms recovered from control mice. The filled in symbols represent male worms and the open symbols female worms. The separate symbols refer to individual experiments (Kr = krad).

It can be seen from Fig. 2 that in terms of number of worms recovered, female worms were not markedly affected by irradiation levels below 8 krad. In contrast male worms were reduced in number by 40% at 4 – 6 krad and were almost totally absent in groups of mice given larvae exposed to 12 krad. A proportion of female worms survived higher doses but at 25 krad no worms were recovered from the gut lumen on day 35.

In Experiment 4 additional groups of mice were killed on days 10, 14 and 21 and Fig. 3 shows the mean number of male and female worms recovered during the five week infection. Again it is clear that whereas male worms were significantly reduced by exposure to 6 krad, female worms tolerated higher doses and the number of worms recovered was not affected until a dose of 12 krad.

Fecal egg counts were carried out 3 – 4 times a week in Experiment 5. The control group infected with normal larvae had a relative fecundity of 15,280, the group given larvae irradiated at 4 krad had a relative fecundity of 5,960 whilst the remaining groups which received larvae exposed to 8, 12, 10, 16 and 18 krad did not produce eggs. It thus
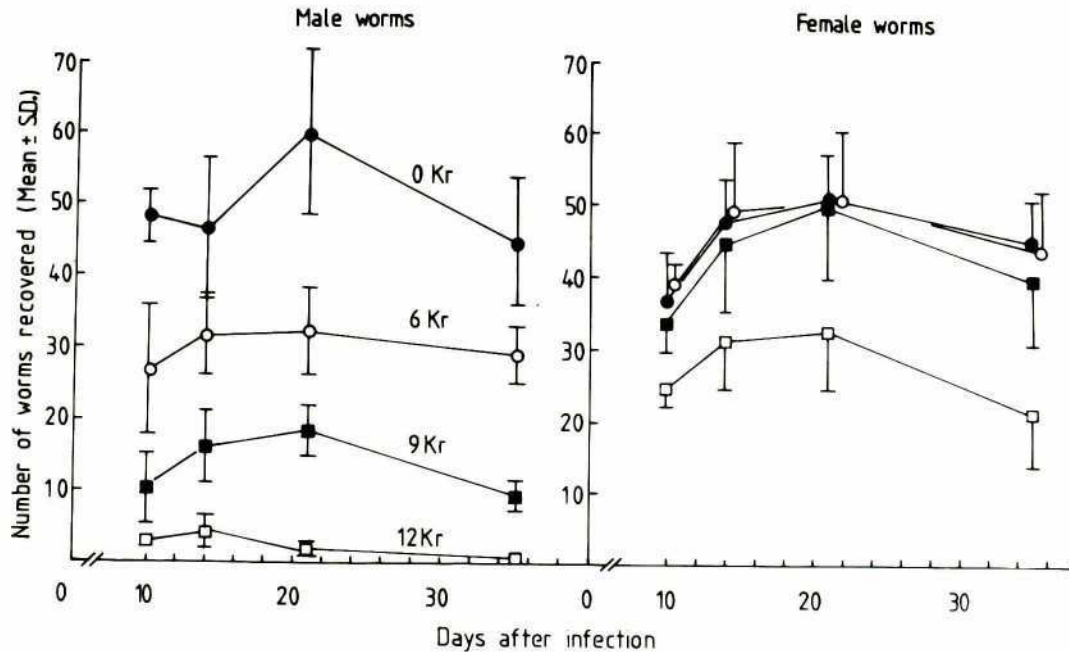
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FIG. 3. Experiment 4. The time-course of the survival of *N. dubius* after infection of the mice with larvae exposed to 0, 6, 9 or 12 krad on day 0. Groups of 4 mice were infected with 100 larvae of *N. dubius* and were killed for worm counts on days 10, 14, 21 or 35 after infection. The results are expressed in terms of mean worm recovery ± standard deviation (Kr = krad).

appears that worm fecundity as assessed by fecal egg counts is affected by doses as low as 4 krad and is totally ablated at a level of 8 krad.

In experiments 1 – 6 changes in worm numbers were monitored during the first five weeks of infection. A single experiment (Experiment 7) was carried out to study the subsequent survival of irradiated and control worms. Thus groups of mice were infected with normal larvae (Group A) or with larvae irradiated at 5, 10 and 15 krad (Groups B, C and D respectively) and were killed for worm counts in groups of 6 – 7 animals 5, 10 and 17 weeks after infection. The results of this experiment are shown in Table 1.

There was a slow but consistent loss of worms from the control group given normal larvae. By the seventeenth week of infection there were 16% fewer worms than on day 35. All the groups infected with irradiated larvae lost a relatively greater proportion of their worm burden, thus the loss in mice infected with larvae exposed to 5, 10 and 15 krad, was 74%, 95% and 100% respectively. Therefore, in contrast to the previous findings that only small changes in worm burden occur during the first 5 weeks of infection in mice infected with larvae irradiated at 5 and 10 krad, the present experiment has demonstrated that their subsequent survival is greatly inferior to that of control worms.

Table 2 shows the results of an experiment (Experiment 8) in which groups of male and female mice of the same age were infected with either 100 normal larvae or 100 larvae irradiated at 5, 10 or 15 krad. All the mice were killed for worm counts on day 35. It is evident from the results that *N. dubius* behaved identically in both male and female mice. There were no significant differences in worm recoveries between groups of male and female mice given identically treated larvae, nor was there a significant difference in the proportion of female worms in the recovered worm burden.

TABLE 1

Experiment 7. The survival of N. dubius over a 4 month period following irradiation of the infective larvae with 5, 10 and 15 krad.

| Dose of radiation to which larvae were exposed | Mean (±S.D.) number of N. dubius recovered[2] | | | | | | | | |
	5 Weeks p.i.[1] ♂worms	♀worms	Total	10 Weeks p.i. ♂worms	♀worms	Total	17 Weeks p.i. ♂worms	♀worms	Total
Group A None	55.4±12.9	53.8±17.5	109.2±10.5	52.7± 7.1	48.8± 9.5	101.5±14.8	46.3±6.8	45.5±9.4	91.8±13.2
Group B 5 krad	32.0± 4.6	45.2± 5.4	77.2± 7.9	19.5±10.9	44.2±13.9	63.7±20.4	0.7 ±1.0	18.5±7.3	20.2±10.2
Group C 10 krad	0	28.8± 6.5	28.8± 6.5	0	6.5± 5.1	6.5± 5.1	0	1.5±3.2	1.5± 3.2
Group D 15 krad	0	8.4± 7.0	8.4± 7.0	0	0	0	0	0	0

[1]Post infection.
[2]The mice were infected with 120 larvae of N. dubius on day 0 and were killed in groups of 5 - 6 animals 5, 10 and 17 weeks after infection.

TABLE 2

Experiment 8. The effect of sex of the host on the survival of N. dubius following irradiation of the infective larvae with 5, 10 and 15 krad.

| Dose of radiation to which larvae were exposed | Male NIH mice | | | Female NIH mice | | |
	Mean	S.D.	%♀	Mean	± S.D.	%♀
Group A None	91.4	15.4	51.2	80.2	± 4.3	56.1
Group B 5 krad	61.4	4.5	67.6	61.4	± 5.5	62.0
Group C 10 krad	30.0	8.6	97.3	28.0	± 2.6	96.2
Group D 15 krad	5.0	3.6	100.0	1.4	± 1.1	100.0

*The mice were infected with 100 larvae of N. dubius on day 0 and were killed in groups of 5 animals on day 35.

TABLE 3

Experiment 9. The survival of N. dubius in mice given different numbers of irradiated or control larvae.

| Number of larvae given | Irradiation of larvae | Mean ± S.D. of N. dubius recovered on day 35* | | | % Control | | |
		♂worms	♀worms	Total	♂worms	♀worms	Total
25	—	8.8± 4.8	10.0± 1.9	18.8± 5.5	—	—	—
25	10 krad	0.2± 0.4	4.8± 4.7	5.0± 4.6	2.3	48.0	26.6
100	—	38.4± 6.7	38.6± 3.0	77.0± 6.4	—	—	—
100	10 krad	0.6± 0.9	28.4± 4.5	29.0± 3.7	1.6	73.6	37.7
300	—	102.0±14.3	148.3±25.2	250.3±34.2	—	—	—
300	10 krad	1.4± 1.7	80.6± 5.5	82.0± 7.0	1.4	54.3	32.8

*The mice were infected with 25, 100 or 300 larvae of N. dubius on day 0 and were killed in groups of 5 animals on day 35.

Table 3 shows the results of an experiment (Experiment 9) in which groups of mice were given either 25, 100 or 300 normal or irradiated (10 krad) larvae. All the groups were killed on day 35 for worm counts.

In the groups infected with 25, 100 and 300 normal larvae, 75%, 77% and 83% respectively of the inoculated larvae were recovered at autopsy. Thus the infectivity of the inoculum was not significantly different when the number of larvae was varied. When the number of worms recovered from groups given irradiated larvae was compared to their respective controls, it was found that they ranged from 26% to 37%. Such a range is consistent with other experiments reported in this paper. The percentage of female worms was, however, more variable (48 – 74%) although when compared to the data in Fig. 2 (Percentage of female worms in mice given 100 larvae irradiated at 10 krad varied from 54 – 72% of control value) these figures appear to be in the expected range. Overall therefore the reduction in worm recovery after irradiation with 10 krad was between 62 and 73% (Experiment 9) and this proportion did not appear to be markedly influenced by the number of larvae in the inoculum.

In order to determine whether an immunological component was responsible for the loss of irradiated worms, several experiments were carried out in which immunosuppressed mice were infected with irradiated larvae. The experimental protocol and the results of one such experiment are shown in Table 4. Groups B, D and F were injected with cortisone every two days from the day shown in Table 4 until autopsy. In addition to the control group given irradiated larvae (Group A), two additional groups of mice (not shown in Table 4) were infected with 150 normal larvae and were killed on days 10 and 35. The number of worms recovered from these groups was 135 ± 10.1 and 138.5 ± 15.1 respectively.

These results show that treatment of the host with cortisone or irradiation did noι increase the number of irradiated larvae recovered from the intestinal lumen. Indeed, in group F significantly fewer worms were recovered on day 35 (P<0.01). It is therefore unlikely that the loss of irradiated larvae is attributable to an immune response or to immune induced arrested development (Behnke and Parish 1979).

DISCUSSION

The experiments described in this paper clearly demonstrated that exposure of the third stage larvae of *N. dubius* to cobalt 60 gamma irradiation had a profound effect on their subsequent development in the host. As the level of irradiation to which the larvae were exposed was increased, so the recovery of worms from the intestinal lumen on day 35 was reduced and this relationship was shown on the semi-logarithmic plot (Fig. 2) to be a complex one. It is clear that there was a marked difference between the effect of gamma radiation on male and on female worms. In both instances, however, the survival curve was characterised by a shoulder at low doses and an exponential component at the higher levels of exposure. The shoulder effect at low doses is known to reflect the number of hits on each organism required to induce a lethal consequence as well as the extent to which the organisms are capable of repairing radiation damage (Coggle, 1971). Whilst, it is not at all clear from the present results how these factors inter-relate in the case of *N. dubius*, the fact that female nematodes are more resistant than male worms to the effects of ionising radiation is well established in the literature (Ciordia and Bizell, 1960; Miller, 1964). Ansari and Singh (1978), for example, recently reported that when the larvae of *Gaigeria pachyscelis* were exposed to 20 krad of cobalt 60 radiation, a predominantly female worm infection was recovered at autopsy, although in contrast to our experiments some male worms survived this level of irradiation. It would appear that above 12 krad no male *N. dubius* were capable of completing development to the 5th stage.

TABLE 4

Experiment 10. The effect of immunosuppression of the host on the survival of irradiated (10 krad) *N. dubius*

Treatment of host	Mean number of *N. dubius* recovered[1]					
	Day 10		Day 21		Day 35	
	Mean ±	S.D.	Mean ±	S.D.	Mean ±	S.D.
Group A None	53.5 ±	6.0	65.5 ±	19.2	54.5 ±	17.8
Group B Cortisone d0[2]	49.8 ±	15.5				
Group C Irradiation 750r d0[3]	43.5 ±	6.5				
Group D Cortisone d10[2]			64.0 ±	11.0		
Group E Irradiation 750r d10[3]			58.3 ±	3.7		
Group F Cortisone d21[2]					33.5 ±	14.5
Group G Irradiation 750r d21[3]					46.0 ±	15.7

[1] The mice were infected with 150 irradiated (10 krad) larvae of *N. dubius* and were killed in groups of 6 animals on day 10, 21 or 35 after infection.
[2] Cortisone was injected every 2 days from the day shown until autopsy.
[3] These mice were exposed to 750r of Cobalt 60 radiation on the day shown.

Whereas some female worms were capable of surviving radiation levels as high as 18 krad, their fecundity was greatly impaired at much lower levels of exposure. Thus in Experiment 5, a dose of 4 krad reduced egg production by 61% and after 8 krad fecundity was totally ablated. Although it is possible that this result is partially attributable to the reduction in the male worm population at this level of exposure, it is more probable that the gonads of both sexes were more severely affected by the lower doses of radiation than the parasites ability to survive.

Our experiments have demonstrated that the capacity of irradiated *N. dubius* to survive *in vivo* for 5 weeks following infection was related to the extent of the damage caused at the time of irradiation and was not dependant on additional host parameters. Thus neither the number of irradiated worms inoculated nor the sex of the host radically altered the sex ratio or proportion of the worms lost as a result of irradiating the larvae.

Since the purpose of these experiments was to provide data on the effects of radiation on *N. dubius* in order to enable the study of the resistance elicited by radiation attenuated worms, it was important to exclude the possibility that an immunological component was responsible for at least a proportion of the loss incurred by the worm population. It is evident from our experiments that neither of the immunosuppressive procedures employed increased the proportion of surviving worms and thus it is highly unlikely that a host response was involved.

Precisely how radiation damaged the worms is unknown and must await further, more detailed studies. However, it is interesting to note here that even at doses of 30 krad when none of the inoculated worms were recovered at autopsy, the larvae must have nevertheless established in the host intestine since a five week infection with such larvae has been shown to elicit 97% protection against a subsequent challenge infection (Behnke and Parish, in preparation). It has been suggested for other species that radiation attenuated larvae still undergo migration through the tissues but encounter problems at the moults to the 4th and adult stages (Ansari and Singh, 1978; Jarrett and Sharp, 1963). The life cycle of *N. dubius* in the host does not involve any extensive migration in the body of the host but the 3rd stage larvae penetrate the intestinal mucosa and develop in the muscularis externa to the 4th stage (Bryant, 1973). It is possible therefore that larvae affected by radiation are unable to complete this moult and hence remain in the intestinal tissues until they eventually die or are destroyed by host cells.

Finally it is intended that, besides providing detailed information about the relationship between the levels of irradiation used and the subsequent survival characteristics of *N. dubius* in the host, the present work draws attention to the suitability of this particular model system for study of radiation damage in nematode parasites. With the data provided herein it should now be possible to determine the nature of irradiation damage incurred by the parasite and to locate the stages at which further development is prevented after specific levels of exposure.

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