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Immunity in Experimental Syphilis

III. Attenuation of Virulent *Treponema pallidum* by γ-Irradiation

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ABSTRACT

MILLER, JAMES N. (University of California School of Medicine, Los Angeles). Immunity in experimental syphilis. III. Attenuation of virulent *Treponema pallidum* by γ-irradiation. J. Bacteriol. **90**:297–301. 1965.—Virulent, freshly isolated cells of *Treponema pallidum* strain Nichols suspended in a 50% rabbit serum-saline solution and exposed to a γ-irradiation dosage of 652,800 r were rendered noninfectious without apparent loss of motility or change in observable morphological and staining characteristics. Although 5×10^7 γ-irradiated organisms failed to elicit an immobilizing antibody response in rabbits, the same organisms retained their capacity to react with classical *T. pallidum*-immobilizing antibody.

Both rabbits and man infected with *Treponema pallidum* develop a relatively high degree of resistance to reinfection during the course of the disease (Magnuson and Rosenau, 1948; Turner and Nelson, 1950; Miller, Fazzan, and Whang, 1963*a*). However, it has not been possible to induce this immunity in rabbits by use of antigens extracted from *T. pallidum* or related organisms (McLeod, 1962; Miller, Whang, and Fazzan, 1963*b*) and *T. pallidum* inactivated by heat, Merthiolate, or lyophilization (Magnuson, Halbert, and Rosenau, 1947; Eagle and Fleischman, 1948; Waring and Fleming, 1951). Although not exhaustive, these investigations have suggested the possibility that more than one antigen may be responsible for conferring protection and that these antigenic components may be irreversibly altered by the inactivation procedures (Miller et al., 1963*b*). Further, since a loss in virulence and corresponding immunogenicity of treponemes freshly isolated from the rabbit appears to be closely associated with a loss in motility, it is conceivable that immunogenic antigens are altered by those procedures which also cause immobilization of the organisms. Thus, the hypothesis was advanced that freshly isolated *T. pallidum* rendered noninfectious but retaining motility might be an effective immunizing agent with antigens remaining intact and complete. It was further postulated that such organisms might be useful in the study of the mechanism whereby immunity develops. In light of this knowledge and speculation, the current series of experiments were carried out to determine whether treponemes suspended in a suitable liquid medium could be modified in this manner by ionizing radiation in the form of γ-rays.

MATERIALS AND METHODS

Preparation of sustaining medium. Blood was obtained from normal New Zealand white rabbits by cardiac puncture; the serum was heated in a water bath (56 C) for 30 min, and then diluted 50% with 0.85% saline. The solution was equilibrated with 95% nitrogen and 5% carbon dioxide, and was incubated at 33 C until ready for use. The medium was prepared fresh the day it was used.

Preparation of treponemal suspensions. Rabbits to be used as a source of treponemes were infected intratesticularly with the Nichols strain of *T. pallidum*. Suspensions were then prepared in the sustaining medium as described by Miller et al. (1963*b*) and adjusted to contain approximately 5×10^7 treponemes per milliliter according to the quantitative method of Magnuson, Eagle, and Fleischman (1948); this is equivalent to 50 spirochetes per high dry dark field with 15× oculars and 40× objectives. Samples (3 ml, containing approximately 1.5×10^8 organisms) to be irradiated, together with the nonirradiated control suspension, were equilibrated with 95% nitrogen and 5% carbon dioxide and transported to the Nuclear Medicine and Radiation Biology Laboratory. Each cotton-stoppered tube containing treponemes to be irradiated was fitted with a sterile screw cap and placed within a larger plastic tube. The suspensions were then exposed to γ-rays emitted from a 10,000 c Co^{60} source at rates varying from 2,205 to 2,550 r per sec, depending upon the amount of Co^{60} decay rate which had occurred on the day the experiment was to be performed.

TABLE 1. *Results* of five experiments comparing the motility of nonirradiated and γ-irradiated Treponema pallidum*

Incubation period	Non-irradiated control	Irradiation dosage (r)		
		5,100–652,800 (serial range)	1,315,600	2,631,200
hr				
0	99–100	97–100	97–100†	0
24	82–100	78–100	0–46	—
48	82–86	76–92	0	—
72	0–48	0–60	—	—
96	0	0	—	—

* Expressed as motility range (%).
† Degree of motility slightly decreased.

Both irradiated and nonirradiated suspensions were returned to the treponemal research laboratory for use in the designed experiment.

Determination of motility. Immediately after irradiation, the percentage of organisms actively motile for both irradiated and nonirradiated suspensions was determined by observing 100 treponemes. A 0.4-ml sample of each suspension was then equilibrated with 95% nitrogen and 5% carbon dioxide, and was incubated at 33 C; motility in each tube was determined daily, based upon the observation of 25 organisms.

Animal injection and criteria for infectivity. Dutch male rabbits with nonreactive Venereal Disease Research Laboratory (VDRL) and *T. pallidum*-immobilizing (TPI) tests were inoculated either intratesticularly or intradermally within 30 min after completion of the irradiation procedure. They were housed in individual cages at an environmental temperature of 68 to 70 F (20 to 21 C). The development of dark-field positive lesions within a 12-week observation period was considered evidence for symptomatic infection. If, however, such lesions failed to develop during this time, the prefemoral, axillary, and popliteal lymph nodes of those rabbits injected intradermally, and the popliteal lymph nodes and testes of those injected intratesticularly, were inoculated into the testes of normal rabbits according to the method described by Miller et al. (1963*b*).

Animals whose nodes or testes, or both, provided dark-field positive lesions during a 13-week observation period were considered to have had asymptomatic infection; failure of lesions to develop was considered indicative of noninfectivity.

Serological testing. Blood for VDRL and TPI testing was obtained at intervals from the marginal ear vein of both the animals injected with the original inocula and those receiving the testes and node suspensions. After the removal of serum, the samples were stored at −20 C until tested. The VDRL slide flocculation test was performed as described in the *Manual of Serologic Tests for Syphilis* (U.S. Public Health Service, 1959). The TPI test was carried out according to the method described by Boak and Miller (1954).

RESULTS

Effect of γ-irradiation on motility. Separate suspensions were irradiated with serial dosages ranging from 5,100 to 2,631,200 r. Comparisons of motility were made with a nonirradiated control suspension immediately after exposure (zero-hour) and daily until motility had declined to 32% or less. As shown in Table 1, on the basis of five experiments, no significant difference was observed when dosages up to and including 652,800 r were employed. However, organisms exposed to 1,315,600 r were slightly less motile than nonirradiated controls; after 24 hr of incubation, a large number of these treponemes were nonmotile. Irradiation dosages of 2,631,200 r rendered the spirochetes nonmotile at the zero-hour observation.

TABLE 2. *Clinical and serological response of rabbits to the intradermal inoculation of 5 × 10^7 γ-irradiated Treponema pallidum*

Irradiation dosage (r)	Symptomatic response (avg days)*	Serological response (avg reciprocal titer)					
		VDRL			TPI		
		4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks
Nonirradiated control	3.0	35	19	5	37	74	155
40,800	12.8	26	15	8	27	87	267
81,600	19.8	18	12	19	NR	17	101
163,200	27.8	0.6	21	3	NR	17	98
326,400	None†	<1‡	NR	NR	NR	NR	NR
652,800	None†	<1‡	NR	NR	NR	NR	NR

* Average of 10 sites (5 × 10^6 *T. pallidum* per site) in each of five animals per group.
† Axillary, prefemoral, and popliteal lymph nodes negative upon transfer.
‡ Reflects the titer produced by a single animal. NR indicates nonreactive.

TABLE 3. *Clinical and serological response of rabbits to the intratesticular inoculation of 5 × 10^7 γ-irradiated Treponema pallidum*

Irradiation dosage (r)	Total no. of rabbits	Clinical response			Serological response (avg reciprocal titer)					
					VDRL			TPI		
		Symptomatic	Asymptomatic	Noninfectious	4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks
Nonirradiated control	10	10[a]	0	0	40	15	6	25	56	227
326,400	18	2[b]	0	16[c]	NR → 1[d]	NR → <1[d]	10[f]	NR	17[f]	65[f]
652,800	19	0	0	19	NR → 1[e]	NR → <1[d]	NR	NR	NR	NR

[a] Average incubation period, 8 days.
[b] Average incubation period, 41 days.
[c] Dark-field and TPI negative for the 13-week period of observation after node and testes transfer.
[d] Two noninfected animals produced titers of 1:1 or less.
[e] Five noninfected animals produced titers of 1:1 or less.
[f] Reflects the average titers produced by the two infected animals.

TABLE 4. *TPI titers of 11 human antisyphilitic sera obtained with nonirradiated and γ-irradiated Treponema pallidum as antigens*

Antiserum no.	TPI titer (reciprocal)	
	Non irradiated antigen (control)	Irradiated antigen (652,800 r)
28745	120	115
28808	62	58
28832	117	115
28835	133	146
28866	108	111
28872	122	120
28875	127	109
28877	128	103
28879	73	63
28881	17	16
28883	118	135

Effect of γ-irradiation on morphological and staining characteristics. No differences were observed by dark-field examination between the morphological structure of nonirradiated spirochetes and those irradiated with serial dosage ranging from 5,100 to 1,315,600 r. However, the suspensions containing organisms exposed to 2,631,200 r were composed entirely of degenerative forms at zero-hour. Spirochetes irradiated with each of these dosages retained their ability to stain by the Fontana-Tribondeau silver impregnation method (Smith and Conant, 1960).

Effect of γ-irradiation on infectivity and antigenicity. The observation that motility as well as morphological and staining characteristics are unaffected by γ-irradiation dosages ranging from 5,100 to 652,800 r led to investigations to determine whether dosages in this range could render virulent *T. pallidum* noninfectious. In preliminary studies, the less sensitive intradermal rather than the intratesticular route was employed because of the relatively larger number of sites which could be utilized. As shown in Table 2, a proportionate delay occurred in the incubation period as the irradiation dosage was increased until, after 12 weeks of observation, lesions failed to develop at those sites receiving *T. pallidum* irradiated with either 326,400 or 652,800 r. Further, the animals in the latter two groups showed no evidence of asymptomatic infection as measured by node transfer.

When the more sensitive intratesticular route of injection was employed, 2 of the 18 animals receiving 5 × 10^7 treponemes γ-irradiated with 326,400 r developed dark-field positive lesions 41 days after inoculation. However, none of the 19 animals injected with the same number of *T. pallidum* exposed to 652,800 r showed signs of either symptomatic or asymptomatic infection during the period of observation (Table 3).

It was of particular interest to note that rabbits which were inoculated with irradiated treponemes and which failed to develop syphilitic lesions produced neither TPI antibody nor significant reagin levels (Table 2 and 3). Further, after node and testes transfer, none of the recipient rabbits produced these antibodies during the 13-week period of observation.

Effect of γ-irradiation on TPI antigen(s). Inasmuch as treponemes rendered noninfectious with relatively high dosages of γ-rays fail to stimulate TPI antibody, studies were designed to determine

whether the TPI antigen or antigens were still present in the treponemes after attenuation with 652,800 r.

A suspension containing *T. pallidum* strain Nichols (5×10^7 per milliliter) was prepared as described from rabbits which had been infected 7 to 10 days previously; one-half of the treponemal suspension was γ-irradiated with 652,800 r, and the remaining half was left untreated. Both samples were then employed as antigens in the TPI test, after being adjusted with the inactivated serum-saline medium to contain 5×10^6 organisms per milliliter for optimal sensitivity, according to the recommendation of Miller, Boak, and Carpenter (1958). No significant quantitative differences were observed between the irradiated and nonirradiated spirochetes; the titers obtained on 11 human antisyphilitic sera were comparable (Table 4). These results provide evidence that the TPI antigen(s) remains intact after irradiation with this dosage.

DISCUSSION

The ability of freshly isolated, γ-irradiated *T. pallidum* cells to retain motility after loss of infectiousness has important immunological implications, despite the failure of these cells to produce an antibody response. In view of the fact that the TPI antigen or antigens remain intact, it appears reasonable to assume that relatively larger numbers of γ-irradiated virulent *T. pallidum* might be capable of stimulating immobilizing antibody. This has particular significance if, as thought by Turner and Nelson (1950), TPI antibody plays an important part in the immune process. The possibility that such organisms may confer protection without the production of immobilizing antibody, however, is suggested from the work of Magnuson, Thompson, and McLeod (1951) and Miller et al. (1963*a*), who have presented evidence that other factors, possibly cellular in nature, may play a role in the development of host resistance. It is significant to note that guinea pigs can be protected against both symptomatic and latent *Leptospira icterohaemorrhagiae* infection by use of γ-irradiated organisms as a vaccine, although "in vitro" agglutination-lysis antibody cannot be detected (Hubbard and Miller, *in press*). The possibility arises, then, that resistance to *T. pallidum* infection in rabbits might be acquired in the same manner without the appearance of immobilizing antibody. This does not necessarily imply that some other humoral mechanism is not operative, particularly since the demonstration by Turner (1939) that immune serum can passively protect rabbits. It seems likely that, although humoral factors may be responsible for protection against symptomatic infection, the organisms become inaccessible to the action of protective antibody after establishing an intracellular residence within lymph nodes and other tissues early in the disease; host factors, presumably operative within immune cells, may be responsible for altering the organisms and maintaining them in a relatively avirulent form. Thus, the animals persist in a state of latency for the remainder of their lives.

Nishihara et al. (1963) have demonstrated that γ-irradiated *Mycobacterium tuberculosis* ($H_{37}Rv$) confers to mice significant immunity. If studies presently underway indicate that a similar protection can be achieved with γ-irradiated *T. pallidum*, a useful tool will become available not only for investigating some of the host factors participating in the immune response, but also for investigating the nature of the antigen or antigens responsible for the development of resistance.

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