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Immunity in Experimental Syphilis

IV. Serological Reactivity of Antigens Extracted from γ-Irradiated *Treponema pallidum* and *Treponema reiteri*

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ABSTRACT

MILLER, JAMES N. (University of California School of Medicine, Los Angeles), J. H. DE BRUIJN, AND J. H. BEKKER. Immunity in experimental syphilis. IV. Serological reactivity of antigens extracted from γ-irradiated *Treponema pallidum* and *Treponema reiteri*. J. Bacteriol. **91**:583–587. 1966.—Ultrasonic lysate preparations extracted from virulent *Treponema pallidum*, Nichols strain, suspensions exposed to 652,800 R of γ-irradiation exhibited a loss in the serological reactivity of their heat-labile antigens; the heat-stable components of both the lysate and residue antigens were unaffected. The activity of heat-stable, cardiolipin *T. pallidum* complement-fixing antigen obtained from similarly irradiated organisms was also unaltered. γ-Irradiation of the cultivable *Treponema reiteri* with dosages as high as 6,500,000 R failed to alter serologically either the heat-labile or heat-stable component of its lipopolysaccharide-protein (Reiter protein) antigen. The reactivity of the lipopolysaccharide portion of the Reiter protein complex with an antiserum to *T. pallidum* Nichols indicates previously unsuspected antigenic differences between the rabbit-adapted Nichols strain of the organism and so-called "wild" human strains of *T. pallidum* in which this antigen is generally absent.

Recent studies showed that virulent *Treponema pallidum*, freshly isolated from the rabbit, is rendered noninfectious without loss of motility after γ-irradiation with 652,800 R (9). Although it was found that treponemes attenuated in this manner retained their capacity to react with *T. pallidum*-immobilizing (TPI) antibody, the reactivity of extractable antigens was not determined. The possibility that such antigens may be altered or destroyed stems from the observation by Leone (7) that γ-irradiation of ovalbumin and human γ-globulin solutions results in a loss of their serological activity. Evidence that a selective inactivation of heat-labile antigens occurs after exposure of *T. pallidum* to 652,800 R would lend further support to the heat-stable and possible polysaccharide nature of TPI antigens, as postulated by D'Alessandro and Zaffiro (3), and thereby direct attention to the possible importance of this class of antigens in the development of resistance.

The present investigation was thus carried out to determine the serological reactivity of heat-labile and heat-stable antigens extracted from suspensions of nonirradiated *T. pallidum*, Nichols strain, and from suspensions of the same organism after exposure to a γ-irradiation dosage of 652,800 R. In an effort to compare these findings with those obtained by use of a cultivable treponeme free of host tissue, similar studies were carried out with γ-irradiated *T. reiteri*.

MATERIALS AND METHODS

Preparation of T. pallidum antigens. Suspensions of virulent *T. pallidum* Nichols containing approximately 5×10^7 organisms per milliliter were prepared in 50% rabbit serum-saline solution and γ-irradiated with 652,800 R as previously described (9). Equal portions of the same suspension were left nonirradiated as a control. Immediately after exposure, the irradiated and nonirradiated suspensions, each with comparable motilities of 94 to 100% and relatively free from tissue debris, were frozen at −20 C. When a total of 6.6×10^9 treponemes in each suspension had been collected, the respective samples were thawed, pooled, and centrifuged at 19,000 × *g* for 90 min. They were washed three times with 10 ml of chilled 0.075 M sodium citrate and homogenized with needle and syringe in 0.14 M saline to give a final concentration of 2.75×10^8 treponemes per milliliter. Both the

irradiated and nonirradiated suspensions were then divided into two equal portions. *T. pallidum* complement-fixing (TPCF) antigens, with and without preliminary acetone and ether extraction, were prepared from one-half of each suspension as described by Portnoy and Magnuson (11).

Three additional antigens were prepared from the remaining portions of both suspensions by use of essentially the procedures employed by D'Alessandro and Dardanoni (2) and modified by De Bruijn (4) for the extraction of lipopolysaccharide-protein [Reiter protein (RP)] antigen from *T. reiteri*. Briefly, after ultrasonic disintegration for 20 min in a Raytheon oscillator (10KH$_2$250 W), the lysates were centrifuged in the cold at 19,000 $\times$ *g* for 60 min. The residues were washed three times with 10 ml of 0.14 M chilled saline and then suspended as a particulate antigen in an amount of saline equivalent to the original volume prior to disintegration. In an effort to concentrate serologically active antigens present in the centrifuged whole lysate preparations, a portion of the supernatant fluid was salted out by the ammonium sulfate-precipitation method described by De Bruijn (4). All antigens were sampled and stored at -20 C.

On the day of testing, a portion of each antigen was heated at 100 C for 60 min. With the exception of the residue preparations, they were then centrifuged at 1,000 $\times$ *g* for 30 min and the supernatant fluids were tested together with the unheated sample. Inasmuch as the serological activity of the residue antigens was presumed to reside in the particles, they were tested without prior centrifugation.

Preparation of T. reiteri lipopolysaccharide-protein (RP) antigens. The organisms were cultured as described by D'Alessandro and Dardanoni (2) and equal samples were γ-irradiated with 10-fold dosages ranging from 6,500 to 6,500,000 R; one portion was left as a nonirradiated control. The suspensions were washed three times with 0.14 M saline, homogenized, and adjusted to contain 2.75 $\times$ 10^8 organisms per milliliter as previously described. Ammonium sulfate-precipitated (RP) antigens were prepared from the ultrasonic lysates according to the method of De Bruijn (4), except that the precipitates were resuspended in an amount of 0.1 M glycine buffer equivalent to one-fourth the original volume of lysate. The preparations were tested in both the unheated and heated state, as already described for *T. pallidum* antigens.

Preparation of antisera. A pool of anti-*T. pallidum* serum was obtained from rabbits infected intratesticularly with *T. pallidum* Nichols 3 to 9 months prior to killing. The serum was lyophilized in 2-ml amounts and was stored at 4 C.

Rabbit anti-RP serum was prepared and stored as described by Miller, Whang, and Fazzan (10).

Sera were inactivated at 61 C for 30 min on the day of testing.

Cardiolipin absorption. Absorption of pooled syphilitic serum was carried out with Venereal Disease Research Laboratory antigen according to the method of Hardy and Nell (6).

Serological procedures. Antigenic activity was determined either by the one-fifth volume complement-fixation (CF) technique by use of 1.5 exact units of complement (1), or by the slide gel-diffusion precipitin method of Mansi (8). The CF test results were based upon readings after a 30-min secondary incubation period. They were considered valid only when the pooled syphilitic serum exhibited a predetermined pattern of reactivity with standard Reiter protein complement-fixing antigen.

RESULTS

Serological reactivity of antigens extracted from γ-irradiated T. pallidum Nichols. Unheated whole lysate and ammonium sulfate-precipitated antigens obtained from irradiated suspensions after ultrasonic treatment exhibited a two- to eightfold decrease in titer when compared with the activity of similar antigens extracted from nonirradiated treponemes (Table 1); the twofold difference in reactivity between the whole lysate preparations was considered significant, inasmuch as it occurred in all dilutions of antigen employed in the experiment. As shown in Table 1, heating of both antigens extracted from the irradiated organisms failed to alter their titers. On the other hand, similarly treated antigens from nonirradiated suspensions showed a significant reduction; the titers obtained with both the whole lysate and ammonium sulfate-precipitated antigens after heating were almost identical to those observed with the corresponding antigens from irradiated treponemes. These results indicate that the heat-labile antigens are inactivated by the irradiation process, and that the serological activity of the heat-stable components are unaffected. The noncardiolipin nature of the heat-stable antigens was inferred as a result of cardiolipin-absorption studies in which titers before and after absorption were found to be identical.

No quantitative differences in serological activity were observed between the unheated ultrasonic residue antigens extracted from nonirradiated and γ-irradiated treponemal suspensions. With antigen dilutions of 1:1 and 1:2, CF activity was produced by serum dilutions of 1:8 and 1:4, respectively. With higher antigen dilutions, the sera were nonreactive. Further, the titers were identical to those obtained with heated antigens. Thus, the residue preparations appeared to contain only a heat-stable component unaffected by the irradiation process. Further, cardiolipin absorption failed to alter the titers.

Inasmuch as TPCF antigen has been considered protein, its serological activity after extraction from γ-irradiated *T. pallidum* suspensions was ascertained. A similar antigen was prepared without the preliminary acetone and ether treatment to determine the irradiation effect upon cardiolipin as well. As shown in Table 2, no sig-

TABLE 1. *Serological reactivity of unheated and heated ultrasonic lysate antigens extracted from nonirradiated and γ-irradiated Treponema pallidum Nichols*

Antigen[a]	Reciprocal dilution of antigen[b]				
	1	2	4	8	16
Nonirradiated TP-WL					
Unheated	256[c]	256	64	32	16
Heated	64	64	32	16	4
Irradiated TP-WL					
Unheated	QNS[d]	128	32	16	4
Heated	QNS	≧64	32	16	4
Nonirradiated TP-AP					
Unheated	128	128	64	32	8
Heated	32	32	16	8	NR[e]
Irradiated TP-AL					
Unheated	QNS	32	16	4	NR
Heated	QNS	32	16	4	NR

[a] WL = whole lysate antigen; AP = ammonium sulfate-precipitated antigen. Irradiation was at 652,800 R, and heating was at 100 C for 60 min.
[b] At an antigen dilution of 1:32, all sera were nonreactive.
[c] Reciprocal of the highest dilution of pooled syphilitic rabbit serum exhibiting 3+ to 4+ CF activity.
[d] Quantity of antigen not sufficient for testing.
[e] Nonreactive.

TABLE 2. *Serological reactivity of unheated and heated TPCF antigens extracted from nonirradiated and γ-irradiated Treponema pallidum Nichols*

Antigen	Reciprocal dilution of antigen			
	2	4	8	16
Nonirradiated TPCF-A[a]				
Unheated	64[b]	64	32	4
Heated	64	64	32	4
Irradiated TPCF-A				
Unheated	64	64	16	4
Heated	64	64	32	4
Nonirradiated TPCF-B[c]				
Unheated	16	8	NR[d]	NR
Heated	16	8	NR	NR
Irradiated TPCF-B				
Unheated	16	8	NR	NR
Heated	16	8	NR	NR

[a] Without preliminary acetone and ether treatment.
[b] Reciprocal of the highest dilution of pooled syphilitic rabbit serum exhibiting 3+ to 4+ CF activity.
[c] With preliminary acetone and ether treatment.
[d] Nonreactive.

TABLE 3. *Serological reactivity of unheated and heated RP antigens extracted from nonirradiated and γ-irradiated Treponema reiteri*

Irradiation dosage	Reciprocal dilution of antigen							
	2	4	8	16	32	64	128	256
Nonirradiated								
Unheated	64*	64	64	64	32	32	16	8
Heated	16	16	16	32	32	16	16	16
6,500 R								
Unheated	64	32	64	32	64	32	32	16
Heated	16	16	16	16	16	16	16	16
65,000 R								
Unheated	64	64	64	64	64	32	32	32
Heated	—	16	16	16	16	16	16	16
650,000 R								
Unheated	64	64	64	64	64	32	16	16
Heated	16	16	16	16	16	16	16	16
6,500,000 R								
Unheated	64	64	64	64	64	32	32	16
Heated	16	16	16	16	16	16	16	16

* Reciprocal of the highest dilution of pooled syphilitic rabbit serum exhibiting 3+ to 4+ CF activity.

nificant difference in reactivity was observed between TPCF antigens extracted from γ-irradiated and nonirradiated treponemal suspensions. Further, their nonprotein nature was suggested by the similar CF titers obtained with heated and unheated preparations. The failure to react with the cardiolipin-absorbed pooled syphilitic serum indicated the "ubiquitous lipid" character of these antigens. Thus, the data indicate that TPCF cardiolipin antigens are unaffected by

γ-irradiation of *T. pallidum* suspensions with 652,800 R.

None of the antigens extracted from *T. pallidum* showed evidence of precipitinogens by the gel-diffusion method in which both rabbit antisyphilitic and anti-RP serum were employed.

Serological reactivity of RP antigens extracted from γ-irradiated T. reiteri. As shown in Table 3, the presence of a heat-labile component in the relatively lower dilutions of antigen was indicated by the reduced titers obtained after heating. However, no significant differences in activity were observed between any of the antigens. Thus, γ-irradiation had no effect upon either the heat-labile protein or heat-stable lipopolysaccharide components.

Precipitinogens, as measured by gel diffusion, were also unaffected by the irradiation process, as evidenced by the same number of lines of identity formed between each of the antigens when tested against anti-RP serum.

DISCUSSION

The evidence presented in this report clearly demonstrates that γ-irradiation of *T. pallidum* Nichols suspensions with 652,800 R results in a serological inactivation of the heat-labile antigens without affecting the heat-stable components. The finding that TPI antigens are unaffected by this dosage of irradiation (9) implies their heat-stable nature. Despite the fact that the role of immobilizing antigens in the immune process is not clear, the possibility arises that heat-stable antigens, possibly polysaccharide in nature, may be significant factors in the development of acquired resistance. If studies in progress indicate that protection can be achieved with treponemes attenuated by γ-irradiation, it would provide additional evidence for the important part played by this class of antigens. In the present investigation, heat-stable antigens have been demonstrated within both the Nichols strain of *T. pallidum* and *T. reiteri*, but their immunogenic role has not as yet been ascertained. The immunology of treponemal infection becomes further complicated by the observation, as confirmed in this study, of common lipopolysaccharide antigen(s) in the rabbit-adapted Nichols strain of *T. pallidum* and in *T. reiteri* (Bekker, De Bruijn, and Miller, Brit. J. Venereal Diseases, *in press*). The absence of this antigen(s) in over 90% of the human strains of *T. pallidum* (5) points to previously unsuspected antigenic differences between the rabbit-adapted and "wild" strains of the organism and stresses the caution which must be exercised in extending to man conclusions based upon studies in the rabbit.

In contrast to the damaging effects of irradiation upon the heat-labile antigens of virulent *T. pallidum*, the serological activity of the heat-labile portion of RP antigen obtained from similarly treated *T. reiteri* was unaffected, even when dosages of 6,500,000 R were employed. Although the reasons for this discrepancy are unknown, one explanation may be that interaction between γ-rays and host tissue present in *T. pallidum* but not *T. reiteri* suspensions produces substances which cause antigenic alterations. It is also possible that some other mechanism may be operative, and that selective inactivation of heat-labile antigens by relatively high dosages of γ-irradiation may occur with other microorganisms.

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