JOURNAL OF BACTERIOLOGY, Nov. 1967, p. 1388-1393
Vol. 94, No. 5

Printed in U.S.A.

Complement and Lysozyme Requirements for Spirochetolysis in Guinea Pig Serum

T. A. NEVIN AND W. J. GUEST

Venereal Disease Research Laboratory, National Communicable Disease Center, Public Health Service, Bureau of Disease Prevention and Environmental Control, Atlanta, Georgia 30333

Received for publication 4 August 1967

During experiments on the immunological immobilization of treponemes, several cultured strains were lysed in the guinea pig serum used as a source of complement. In further studies employing *Borrelia vincentii*, lysis, observed by dark-field, appeared as swelling and some beading of the cells. Complete disruption eventuated. Untreated guinea pig serum was strongly lytic, whereas little or no lysis occurred in bentonite-adsorbed samples. Activity was restored to these samples by adding commercially obtained crystalline egg-white lysozyme. Serum samples which had been heated, absorbed with aggregates of human γ-globulin, or treated with ethylenediaminetetraacetate to remove, or inactivate, complement also lost spirochetolytic activity.

During experiments on the immobilization of cultured treponemes in sera from syphilitic humans (4), several of the laboratory strains were readily disrupted in suspensions containing active guinea pig serum but not in those containing heat-inactivated serum. In addition, it has been known for some time that the inclusion of lysozyme in reaction mixtures enhances the immobilization of *Treponema pallidum* in diagnostic testing (6, 9). Since complement is needed in the *T. Pallidum* Immobilization (TPI) test (11), it seemed possible that the above-noted spirochetolysis (3) required both active complement and lysozyme. Among the several available cultures, *Borrelia vincentii* (N-9) proved most susceptible to lysis and was used for further investigations.

MATERIALS AND METHODS

B. vincentii was cultivated in a partially defined medium (12) in which a rabbit serum fraction was substituted for ascitic fluid. The serum fraction was insoluble in acetone (4 volumes, 25 C), and soluble in water at *p*H 4.7 (13). It was further purified by a combination of gel filtration and preparative thin-layer chromatography (H. Russell and T. A. Nevin, Bacteriol. Proc., p. 112, 1964; H. Russel et al., Bacteriol. Proc., p. 76, 1965; and *unpublished data*). Other cultures employed were the Nichols, Noguchi, Kazan-2, Reiter, and English Reiter strains, reportedly avirulent cultures of *T. pallidum*, and the N-39 and MRB strains of *T. microdentium*. These were grown routinely in NIH Thioglycollate Broth (Difco) enriched with heated (60 C, 2 hr) rabbit serum (10%, v/v). The cells were harvested by centrifugation, then suspended in enough 0.066 M disodium phosphate buffer (*p*H 6.25) to yield 15 × 10^6 cells/ml.

Fresh guinea pig blood was obtained from young, healthy animals by cardiac puncture; the cells were removed by centrifugation, and the sera were pooled and stored at −20 C. Three lots, obtained from the Venereal Disease Research Laboratory testing section, had been stored at −70 C. The guinea pig serum complement level was determined by titration against sensitized sheep erythrocytes, and the complement was absorbed with human γ-globulin aggregates (0.5 ml of packed, wet particles per ml of serum; at 4 C for 16 hr) (2). Samples of serum, without sorbent, were carried in parallel to serve as controls. Complement was also inactivated by heating at 56 C for 30 min, or by the addition of 25 μmoles of sodium ethylenediaminetetraacetate (EDTA) per ml of serum sample. EDTA inactivation of control samples was prevented by the addition of equimolar amounts of both EDTA and $CaCl_2$ (7). The lysozyme activity of the guinea pig serum was assayed with suspensions of *Micrococcus lysodeikticus* by the method of Smolelis and Hartsell (14), and was reduced by adsorption with bentonite [5 mg of bentonite (*p*H 7.7) per ml of serum] at 0 C, as described by Wardlaw (16). Each pool of serum and each sample which had been treated by any of the means described was titrated for hemolytic activity before use (15). Crystalline egg-white lysozyme was obtained commercially (Mann Research Laboratories, New York, N.Y.). When used, the crystalline enzyme was added to bentonite-adsorbed serum samples in amounts equal to those lost during adsorption. Similar amounts of enzyme, with 0.066 M phosphate buffer replacing the serum, were used as controls. A human serum, from an individual having secondary syphilis at the time of bleeding, was obtained from the Venereal Disease Research Laboratory Serum Bank.

The method for determining motility was essentially as described for the TPI test (15), as modified by Guest et al. (4), for studying cultured spirochetes. Percentage of lysis was determined from the number of intact cells per 100 still identifiable spirochetes after incubation for about 1 hr in a mixture containing 0.3 ml of a washed cell suspension and 0.2 ml of guinea pig serum. Dark-field preparations showing the lytic reaction were photographed at × 2,000 on Kodak contrast process panchromatic film, with a 2-min exposure time.

RESULTS

Neither immobilization nor lysis occurred when an actively motile suspension of *B. vincentii* was added to a mixture containing 0.05 ml of heat-inactivated (56 C, 30 min) serum from a patient with secondary syphilis and 0.2 ml of similarly inactivated guinea pig serum. However, when graded amounts of active guinea pig serum, adjusted to contain 1,000 units of complement per ml, were added to the human serum, almost complete immobilization and marked lysis occurred in mixtures containing 0.1 ml (Table 1). Averaged data from 10 experiments are included. Significant levels of immobilization and lysis occurred also when the unheated guinea pig serum was used separately. In separate experiments, there was no evidence of antispirochetal activity in 0.05-ml amounts of sera from nonsyphilitic humans.

Figure 1 depicts the lytic reaction in guinea pig serum from 0 time to about 1 hr. Figure 1A shows a chain of intact cells with some early "beading" (about 5 to 10 min). The swollen, hollow appearance in Fig. 1B is frequently seen (about 20 to 30 min) as an intermediate degree of disruption. Almost complete disruption with the development of small sphere-like structures (about 40 to 50 min) may be seen in Fig. 1 C. A portion of the spirochete is still recognizable. Figure 1D (about 60 to 70 min) represents the last recognizable stage of the reaction. There remains a suggestion of spirochetal morphology in the distribution of the sphere-like structures.

TABLE 1. *Effect of graded decreases in the amounts of unheated guinea pig serum[a] on the immobilization[b] of Borrelia vincentii in human "secondary syphilis" serum and without added human serum*

Condition	Amt (ml) of guinea pig serum				
	0.20	0.15	0.10	0.05	0[c]
With 0.05 ml of human serum	84[d]	96	96	50	0
Without human serum	79	56	48	8	0

[a] A 0.2-ml amount of serum contained 200 units of complement.

[b] Lysed, but still recognizable, organisms were included in the calculation of percentage of immobilization.

[c] A 0.2-ml amount of heated (56 C, 30 min) guinea pig serum.

[d] Figures represent percentage of immobilization.

Unheated guinea pig serum lost its spirochetolytic activity when treated with bentonite in the cold (6). Although the hemolytic titers of serum samples were reduced by as much as 50 to 60% during bentonite treatment, sufficient activity remained (about 450 units/ml) to bring about some spirochetolysis when a bentonite-adsorbable substance was not involved (Table 2). Upon the addition of sufficient crystalline egg-white lysozyme to bentonite-adsorbed sera to approximate the amounts indicated by assays of the original samples, spirochetolysis was restored to unheated, but not to heated (56 C, 30 min) samples. The lysozyme by itself had no effect on the organisms (Table 2).

Similarly, samples of guinea pig serum to which EDTA had been added (7), or which had been absorbed with aggregates of human γ-globulin (2), were no longer actively spirochetolytic, nor did these samples have high hemolytic titers. However, samples to which equimolar amounts of EDTA and $CaCl_2$ had been added were both spirochetolytic and hemolytic at levels comparable with the controls (Table 3).

When several laboratory strains of spirochetes were tested in the manner described, they did not appear equally susceptible to lysis, and one strain appeared completely resistant. These data are summarized in Table 4. All of the strains tested have been cultured four times in the *B. vincentii* medium (12) supplemented with heated (60 C, 2 hr) rabbit serum. Therefore, lysis of cultures of a readily lysed strain, Kazan-2, grown in NIH Thioglycollate Broth and in the *B. vincentii* medium, was compared. The results (Table 5) indicate that growth of this strain of cultured *Treponema* in the *B. vincentii* medium with added rabbit serum rendered the cells less susceptible to lysis in guinea pig serum than were cells cultivated in the NIH Thioglycollate Broth with added rabbit serum. It does not seem likely that the heated rabbit serum was reactive in the lytic system, since cells of *B. vincentii* in serum-enriched (10%, v/v) NIH Thioglycollate Broth were lysed as readily as those grown in the *B. vincentii* medium. The compound, or compounds, in the *B. vincentii* medium, which effected the observed difference in susceptibility to spirochetolysis, have not been identified.

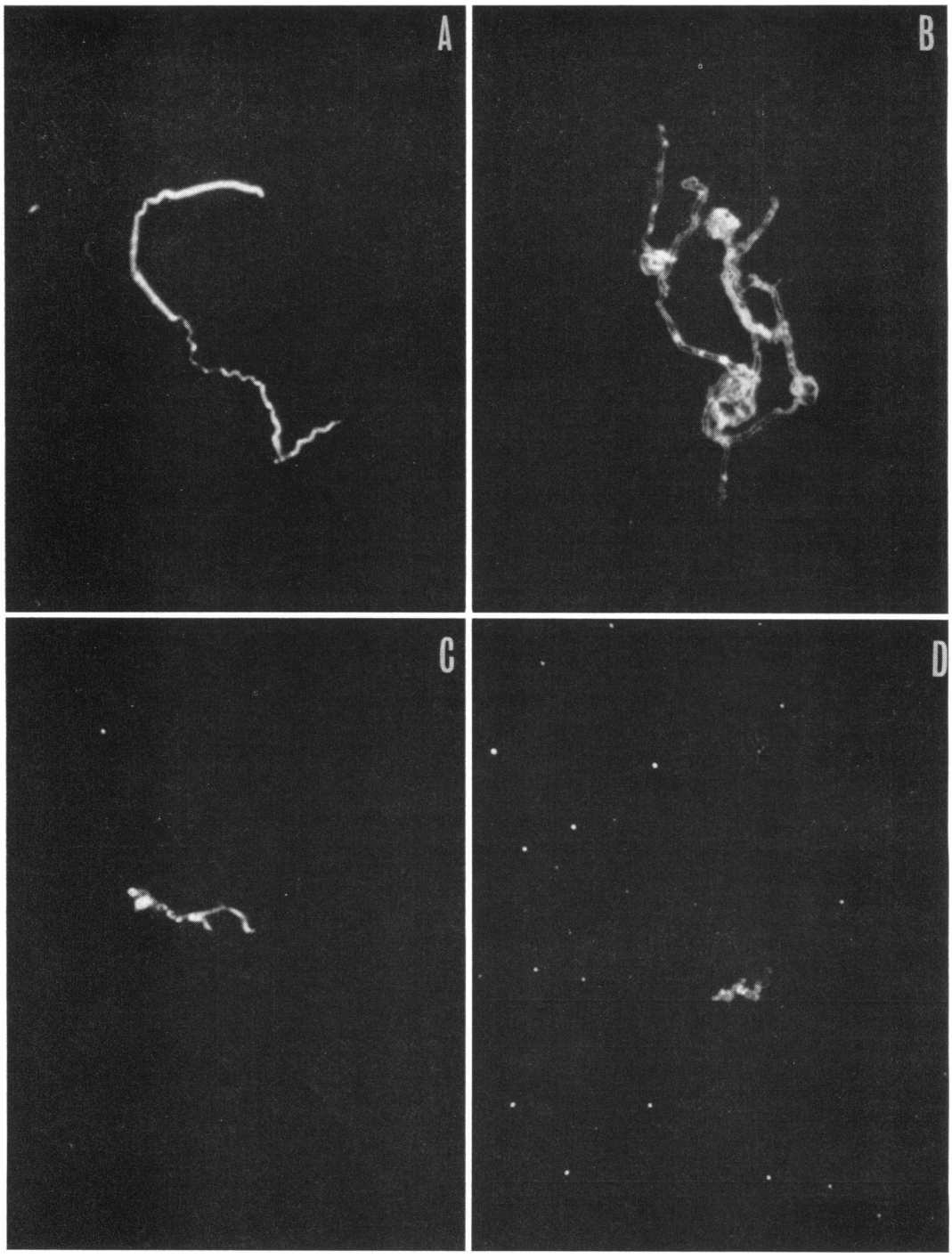

FIG. 1. *Disintegration of Borrelia vincentii with time, as a result of the action of guinea pig serum. (A) At 5 to 10 min, a chain of intact spirochetes. (B) At 20 to 30 min, several spirochetes which appear swollen and hollowed out. Some "beading" and terminal distention are also evident. (C) At 40 to 50 min, partial degeneration into sphere-like bodies. Some of the spirochete remains intact. (D) At 60 to 70 min, complete degeneration into sphere-like bodies. Only a suggestion of spirochetal morphology remains.*

TABLE 2. *Requirement for a heat-labile component and lysozyme for the lysis of Borrelia vincentii in guinea pig serum*

Serum	Intact cells (%)	Amt (μg) of lysozyme/ml of serum	Complement (units/ml)
Bentonite-adsorbed	100	0	440
Bentonite-adsorbed heated	97	0	0
Bentonite-adsorbed, lysozyme added	19	2.9	440
Bentonite-adsorbed, lysozyme added, heated	98	2.9	0
Unadsorbed	9	2.9	1,057
Unadsorbed, heated	99	2.9	0
Lysozyme in buffer	100		
Cells in buffer	100		

DISCUSSION

As was suggested in a prior publication (4), and as indicated by the data in Table 1, at least two antispirochetal reactions were observed when actively motile suspensions of *B. vincentii* were studied by a modified TPI reaction. One of them was brought about by the human serum, since a much lower percentage of organisms remained motile when this serum and intermediate levels of guinea pig serum were used together. The second, a lytic reaction, occurred in the mixture of human and guinea pig sera and when the guinea pig serum was used separately. Evidently (Table 2), a heat-labile serum component and lysozyme were both required. It is probable that there was a suitable sensitizing antibody in the guinea pig serum, since the sera of many animals are known to react in various ways with a wide variety of bacteria (17).

To identify further the heat-labile serum component, unheated serum samples were treated with EDTA, with equimolar amounts of EDTA and $CaCl_2$ (7), or were absorbed with aggregates of human γ-globulin in the cold. Each of the methods used demonstrated that marked spirochetolysis occurred only when the serum samples were actively hemolytic (Table 3). It seems reasonable to assume, therefore, that the heat-labile component was complement.

The varied levels of susceptibility of the several strains to spirochetolysis (Table 4) have several possible explanations. It may be suggested, for example, that there were qualitative or quantitative differences, or both, in the content of natural sensitizing substances in the guinea pig sera. *Lactobacillus plantarum*, however, when cultivated in a pantothenic acid-deficient medium, was more susceptible to lysozyme action than parallel cultures grown in medium adequately supplied with the vitamin (5). Similarly, the Kazan-2 strain of *Treponema* was readily lysed when grown in serum-enriched NIH Thioglycollate Broth, but resisted lysis when grown in the *B. vincentii* medium (Table 5). Thus, it is at least equally probable that susceptibility to spirochetolysis may be related to the adequacy of the culture medium. The continued susceptibility of *B. vincentii* to lysis, when cultured in either medium, may, therefore, reflect inadequacies of both media for this spirochete. It is also conceivable that the difference in susceptibility to lysis is an expression of generic differences between the *Borrelia* and the *Treponema*. Additional observations of such differences have been discussed by Muschel (10).

A complement requiring lysozyme reaction against *Escherichia coli B* has been described by Amano *et al.* (1), who adsorbed fresh human serum with bentonite and reduced its bacteriolytic action. Bacteriolysis was restored by the addition of egg-white lysozyme. Subsequently, Wardlaw (16), by applying bentonite-adsorption and heat-inactivation methods to human serum, indicated that both complement and lysozyme were necessary for the lysis of certain strains of *E. coli*. Glynn and Milne (3) confirmed Wardlaw's work by inactivating the enzyme with antihuman-lysozyme serum. Apparently, similar requirements obtain for guinea pig serum spirochetolysis (Tables 2 and 3).

The probable significance of serum spirochetolysis in natural immunity is, recognizably, a matter for speculation. It is interesting, however, that Metzger (8) observed increasing levels of lysozyme-like activity in the edema fluid as an orchitis developed in rabbits after intratesticular injection with suspensions of virulent *T. pallidum*. A similar increase did not occur in

TABLE 3. *Spirochetolysis and hemolysis in guinea pig serum treated to absorb or inactivate complement*

Serum sample	Intact spirochetes (%)	Complement (hemolytic units/ml)
Untreated	1	1,354
Heat inactivated	97	0
Absorbed[a] with human globulin aggregates	98	25
EDTA added[b]	88	0
EDTA and Ca^{++} added[b]	4	1,280
Control[a]	5	1,371

[a] At 4 C, 16 hr.

[b] Amount of 25 μmoles per ml of serum.

TABLE 4. *Susceptibility of various laboratory strains of treponemes to lysis in the lysozyme-complement system*

Guinea pig serum	Per cent intact cells after 1 hr						
	Noguchi	Nichols	Kazan-2	Reiter	English Reiter	N-39	MRB
Unadsorbed	1	2	5	52	53	8	100
Adsorbed[a], lysozyme added	19	28	12	61	48	53	100
Adsorbed[a]	66	70	77	87	75	72	100
Heated	85	86	94	75	94	99	100
Lysozyme only	100	100	100	99	100	100	100
Buffer only	99	100	100	100	100	100	100

[a] Adsorbed with bentonite; complement level about 450 to 475 units ml.

TABLE 5. *Variation of Kazan-2 strain in susceptibility to lysis as related to culture medium*

Guinea pig serum lot no.	Medium enriched with rabbit serum	Per cent intact cells	
		Test	Heated serum control
396	NIH Thioglycollate Broth[a]	0	100
	Borrelia vincentii[b]	91	100
397	NIH Thioglycollate Broth	0	100
	B. vincentii	84	100
398	NIH Thioglycollate Broth	13	100
	B. vincentii	83	100

[a] Difco.
[b] Reference 12.

the serum lysozyme level of the infected animals. Thus, a required component for the lytic reaction is known to accumulate in the area of infection, suggesting a possible localized inflammatory response.

ACKNOWLEDGMENT

We take this opportunity to thank Mrs. Leslie C. Logan who furnished the γ-globulin aggregates used.

LITERATURE CITED

1. AMANO, T., S. INAI, Y. SEKI, S. KASHIBA, A. FUJIKAWA, AND S. NISHIMURA. 1954. Studies on the immune bacteriolysis. I. Accelerating effect on the immunobacteriolysis by lysozyme-like substance of leucocytes and egg-white lysozyme. Med. J. Osaka Univ. **4**:401–418.
2. EDELMAN, C. M., H. G. KUNKEL, AND E. C. FRANKLIN. 1958. Interaction of the rheumatoid factor with antigenantibody complexes and aggregated gammaglobulin. J. Exptl. Med. **108**:105–120.
3. GLYNN, A. A., AND C. M. MILNE. 1965. Lysozyme and immune bacteriolysis. Nature **207**:1309–1310.
4. GUEST, W. J., T. A. NEVIN, M. L. THOMAS, AND J. A. ADAMS. 1967. Immobilization of certain cultured treponemes in sera from syphilitic humans. J. Bacteriol. **93**:1190–1191.
5. HOLDEN, J. T., AND J. H. A. VAN BALGOOY. 1962. Effect of nutritional and physiological factors on the reaction between *Lactobacillus plantarum* and muramidase. Biochem. Biophys. Res. Commun. **19**:401–406.
6. KENT, J. F., AND J. B. DEWEERDT. 1963. Enhancement by lysozyme of the sensitivity of *Treponema pallidum* immobilization test. Brit. J. Veneral Diseases **39**:37–40.
7. LEVINE, L., K. M. COWAN, A. G. OSLER, AND M. M. MAYER. 1953. Studies on the role of Ca^{++} and Mg^{++} in complement fixation and immune hemolysis. II. The essential role of calcium in complement fixation. J. Immunol. **71**:367–373.
8. METZGER, M. 1963. Lysozyme activity of rabbit sera and testicular juice during the development of syphilitic lesions. Arch. Immunol. Therapiae Exptl. **11**:608–613.
9. METZGER, M., P. H. HARDY, JR., AND E. E. NELL. 1961. Influence of lysozyme on the treponema immobilization reaction. Am. J. Hyg. **73**:236–244.

10. MUSCHEL, L. H. 1964. Immune bactericidal and bacteriolytic reactions. Ciba Found. Symp. Complement, p. 166–174.
11. NELSON, R. A., AND M. M. MAYER. 1949. The immobilization of *Treponema pallidum in vitro* by antibody in syphilitic infection. J. Exptl. Med. **89**:369–393.
12. NEVIN, T. A., AND E. G. HAMPP. 1959. A partially defined medium for the cultivation of *Borrelia vincentii*, J. Bacteriol. **78**:263–266.
13. NEVIN, T. A., AND H. V. JORDAN. 1961. Natural or derived materials which replace ascitic fluid for the growth of an oral spirochete. J. Dental Res. **40**:622.
14. SMOLELIS, A. N., AND S. E. HARTSELL. 1949. The determination of lysozyme. J. Bacteriol. **58**:731–736.
15. U.S. PUBLIC HEALTH SERVICE. 1964. Serolic tests for syphilis. U.S. Public Health Serv. Publ. 411.
16. WARDLAW, A. C. 1962. The complement dependent bacteriolytic activity of normal human serum. I. The effect of pH and ionic strength and the role of lysozyme. J. Exptl. Med. **115**: 1231–1249.
17. WILSON, G. S., AND A. A. MILES. 1964. *Topley and Wilson's Principles of Bacteriology and Immunity*, p. 1315–1339. vol. 2, 5th ed. The Williams & Wilkins Co., Baltimore.