Leptospiral Attachment to Four Structural Components of Extracellular Matrix

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ABSTRACT. The attachment of virulent *Leptospira interrogans* to four structural components of extracellular matrix (ECM), fibronectin, collagen, laminin and hyaluronic acid was investigated using component-coated coverslips for each. Number of attached organisms increased with increasing concentrations of each component, but heat-or formalin-inactivated organisms did not attach to any of these components. The extent of leptospiral attachment to fibronectin, collagen and laminin was respectively more than that to hyaluronic acid. Inhibitory effects on leptospiral attachment of ECM were observed in the presence of soluble fibronectin, collagen and laminin. Hyaluronic acid, however, did not affect the attachment. Also, pretreatment of ECM with antifibronectin, anticollagen or antilaminin decreased the number of attached organisms. Highly virulent lines of leptospires attached more readily to fibronectin, collagen and laminin than did avirulent lines of the same strains, but leptospiral attachment to hyaluronic acid was not correlated to virulence. These findings *in vitro* may suggest that virulent *L. interrogans* adheres to host tissues through fibronectin, collagen and laminin *in vivo*.—**KEY WORDS:** attachment, extracellular matrix, leptospira.

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The attachment of bacteria to host mammalian cells or tissues may be an essential step in the initiation of bacterial infection [2]. Concerning the attachment of spirochetes, the attachment of *Treponema pallidum* to fibronectin has been studied in detail [6, 7, 13, 17–20]. Recent studies have implicated three treponemal outer envelope proteins as receptor binding proteins to fibronectin [13], and the common functional peptide to each protein has been identified [18]. Furthermore, it was reported that fibronectin tetrapeptide arg-gly-asp-ser (RGDS) is a target for treponemal cytaadherence [19].

Little information is available, however, on the mechanisms of leptospiral attachment. Virulent lines of leptospires attached more effectively to cultured cells than did avirulent lines of the same strains, suggesting that a correlation exists between virulence and the extent of attachment [21, 24]. We previously showed that virulent leptos-

pires also attached to the extracellular matrix (ECM) that existed between cells and tissues [9]. It is known that ECM is composed of fibronectin, collagen, laminin and hyaluronic acid [4, 10, 15, 16, 23]. These observations prompted a further investigation of leptospiral attachment to individual components of ECM to see if a similar mechanism as that to treponemes exists with leptospires.

In this paper, we demonstrated the attachment of leptospires to purified structural components making up ECM.

MATERIALS AND METHODS

Leptospires: The strains used were highly virulent, intermediately virulent and avirulent lines of *Leptospira interrogans* serovar *copenhageni* Shibaura, *Canicola* Moulton and *Pomona* MLS. LD₅₀ of the strains determined by intraperitoneal inoculation is shown in Table 1. The history of passage of

Table 1. Virulence of strains used

Strains and lines		Virulence	
		Designation	LD ₅₀ in hamsters
<i>copenhageni</i>			
Shibaura	Line No.1	Highly virulent	10 ⁴
„	Line No.3	Avirulent	>10 ⁸
„	Line No.5	Avirulent	>10 ⁸
M 20		Avirulent	>10 ⁸
<i>canicola</i>			
Moulton		Highly virulent	10 ⁴
„		Intermediately virulent	>10 ⁸
Hond Utrecht IV		Avirulent	>10 ⁸
<i>pomona</i>			
MLS		Highly virulent	10 ⁸
„		Intermediately virulent	>10 ⁸
Pomona		Avirulent	>10 ⁸

these strains is as follows. Serovar *copenhageni* Shibaura line No. 1 was passaged through guinea pigs every 2 months and shown to be lethal to them [25]. Lines No. 3, a streptomycin resistant mutant [14], and No.5, derived from line No. 1, were passaged through media for more than 12 or 16 years, respectively, and proved to be avirulent to guinea pigs [25]. Highly virulent lines of serovar *canicola* Moulton and *pomona* MLS were passaged through hamsters several times a year and shown to be hamster-lethal [21, 22]. These highly virulent lines of *canicola* Moulton and *pomona* MLS were passaged through media for more than 6 years, after which they showed leptospiremia in hamsters but did not kill them and thus were considered intermediate in virulence. Each reference strain of the same serovars was also used (Table 1); they were avirulent, as suggested by the finding that neither leptospiremia (guinea pig and hamster) nor fever (guinea pigs) could be proved for 7 days after intraperitoneal exposure.

Leptospires were grown for 4 days at 30°C

in 0.2% tryptose phosphate broth (Difco Laboratories, Detroit, Michigan, USA) containing 10% normal rabbit serum [3]. Highly virulent lines used for attachment tests were the 2nd cultures of the heart blood which was obtained from experimentally infected guinea pigs or hamsters which were moribund. Cultures were inactivated either by heating at 56°C for 30 min or by treating with 0.2% formalin for 30 min.

Leptospiral attachment to component-coated coverslips: Attachment tests to component-coated coverslips were performed as follows. Purified fibronectin, collagen, laminin and hyaluronic acid were solubilized with phosphate buffered saline (PBS, pH7.2). A 10 μ l volume of each of the respective component solutions was spread evenly over the entire glass surface and allowed to air dry at room temperature. Indirect immunofluorescence assay demonstrated the uniformity of protein-coating on coverslips. A volume of 1 ml of leptospores at 10⁸ organisms/ml was added to the component-coated coverslips placed in 24-well multiplates. The plates were incubated for 2 hr at 30°C. The condition was determined after some preliminary experiments. After incubation, the coverslips were drained, gently rinsed 7–8 times in PBS to remove non adhering leptospores and placed on slides. The degree of attachment was measured by counting the number of leptospores that were attached to 20 randomly selected individual fields at a magnification of $\times 600$ under dark-field microscopy. The average number of attached leptospores/field with standard error was calculated and each result was compared by Student's *t* test. Experiments were repeated at least four times.

Attachment of leptospores to ECM: Mouse fibroblast (L929) cells were used for the isolation of ECM. The cells were seeded at a density of 1.0–1.5 $\times 10^5$ /ml in dishes (6 cm in

diameter) containing coverslips, and grown to 80–90% confluence for 4 days in Eagle's medium (MEM) (Nissui Seiyaku Co., Tokyo, Japan) containing 10% calf serum in an atmosphere of 5% CO₂ in air at 37°C. ECM preparations were obtained from the cell cultures on coverslips by treatment with 0.5% Triton X-100 for 10 min at room temperature. Coverslips were then washed three times with PBS before use. The coverslips were incubated and the degree of attachment was measured as described above. For some experiments, leptospires were also pretreated with different concentrations of soluble fibronectin, collagen, laminin, hyaluronic acid or synthetic tetrapeptide arg-gly-asp-ser (RGDS) at room temperature for 30 min prior to incubation with test coverslips.

Blockage of attachment of leptospires to ECM with antifibronectin, anticollagen and antilaminin antisera: To examine the blockage of attachment by antibodies to each purified matrix components, ECM was treated with up to 10 μ l of antiserum to fibronectin, collagen or laminin for overnight at 4°C prior to addition of leptospires. Three-times treatment each with 10 μ l of antiserum against fibronectin, collagen or laminin was also tested. ECM was washed three times with PBS before each treatment with 10 μ l of antiserum. The presence of fibronectin, collagen and laminin in ECM was confirmed using the enzyme-linked immunosorbent assay according to the method of Thomas *et al.* [20].

Preparation of antisera: Rabbits were immunized with an initial injection of 1 mg of purified type I collagen emulsified in Freund's complete adjuvant. Booster injections of 500 μ g were given two additional times at 14-day intervals. Rabbits were bled 4 days after the last booster inoculation. Anticollagen antibodies were measured by the enzyme-linked immunosorbent assay with collagen-coated microtiter wells. Anti-

serum to fibronectin was obtained from UCB-Bioproducts S. A. (Belgium) and antiserum to laminin from E-Y Laboratories, Inc. (San Mateo, California, USA). Pooled normal rabbit sera served as controls.

Chemicals: Purified fibronectin and collagen (type I) were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Type I collagen is known to be the main collagen in animal skin. Laminin was obtained from Collaborative Research, Inc. (Waltham, Massachusetts, USA) and hyaluronic acid from Laboratories Sarget (Genay, France). The coverslips were freshly coated with samples just before each experiment. RGDS peptide was purchased from Peninsula Laboratories (Belmont, California, USA).

RESULTS

Optimal conditions for attachment of leptospires were first determined, using *copenhageni* Shibaura line No. 1 and fibronectin (400 μ g). Leptospires attached to fibronectin was large in number at 2 hr at 30°C and 37°C but small at 4°C. The number of attached leptospires to fibronectin reached a maximum at 2 hr and remained unchanged until 4 hr. Based on the above findings, incubation in subsequent experiments was carried out at 30°C (optimal temperature for growth of leptospires) for 2 hr.

To determine whether leptospires attach directly to individual components of extracellular matrix, leptospires were added to coverslips precoated with different concentrations of purified fibronectin, collagen, laminin and hyaluronic acid, respectively. Fig. 1a is a dark-field view showing the attachment of many leptospires to collagen on the coverslips. Leptospires also attached similarly to other structural components of ECM, fibronectin, laminin and hyaluronic

Fig. 1. Dark-field view ($\times 600$) of attachment of virulent line (line No. 1) of *L. interrogans* serovar *copenhageni* Shibaura to collagen ($400\ \mu\text{g}$)-coated coverslips (a) and its comparison with the lack of attachment of leptospires to albumin-coated coverslips (b). Some leptospires were out of focus because they were still moving after attachment.

acid. In contrast, leptospires did not attach to coverslips coated with bovine serum albumin (Fig. 1b) or uncoated coverslips. Fig. 2 gives one of the typical results of attachment of *copenhageni* Shibaura line No. 1 to these four structural components. In all cases, the number of attached organisms increased with increasing concentrations of each component. The number of attached leptospires to hyaluronic acid was less than that to other components. Repeated experiments gave similar results. Heat- or formalin-inactivated virulent leptospires did not attach to any of these components. This result showed that leptospires must be viable for attachment to occur.

We next attempted to investigate the effect of each soluble component on leptospiral attachment to ECM (Fig. 3). The

Fig. 2. Attachment of viable versus heat- or formalin-inactivated *L. interrogans* serovar *copenhageni* Shibaura line No. 1 to collagen (a), fibronectin (b), laminin (c) and hyaluronic acid (d). Symbols: viable (●), heat-inactivated (□) and formalin-inactivated (△) leptospires.

number of attached leptospires to ECM was 68.5/field in the absence of soluble components. The presence of $50\ \mu\text{g}$ of soluble fibronectin resulted in 2.1-fold higher level of leptospiral attachment to ECM. When $400\ \mu\text{g}$ of fibronectin was added, inhibition of leptospiral attachment to ECM was observed (53%). The presence of soluble collagen similarly showed enhancement (up to 1.7-fold higher) at low-concentrations (50 – $100\ \mu\text{g}$) and inhibition (52%) at a high concentration ($400\ \mu\text{g}$). Addition of soluble laminin resulted only in a decrease in the adhesion of leptospires. No effect was observed when hyaluronic acid was added. Since the peptide of fibronectin cell-binding domain containing RGDS sequence has been shown to inhibit the adherence of *Treponema pallidum* [19], inhibitory effect of this peptide on leptospiral attachment

was also tested. As shown in Fig. 3, RGDS peptide did not effect on leptospiral attachment of ECM.

The blockage of leptospiral attachment by antibodies to each purified matrix components was then examined. The pretreatment of ECM with 10 μ l of antifibronectin antiserum inhibited attachment by 31.3% (Table 2). Similar inhibitory effects were

Fig. 3. Effect of soluble components on leptospiral attachment to ECM. Each symbol and vertical bar represents a mean $\pm$ S. E. of four separate experiments. Symbols: collagen (●), fibronectin (▲), laminin (○), hyaluronic acid (△) and RGDS peptide (□).

observed in the preexposure of ECM with 10 μ l of anticollagen or antilaminin antibodies (32.5% and 28.3%). Preincubation of ECM with 10 μ l of a mixture of antifibronectin, anticollagen and antilaminin sera produced 56.8% inhibition of leptospiral attachment to ECM. Antiserum less than 10 μ l gave less inhibition. Three-times treatment with 10 μ l of each antiserum or 10 μ l of the mixture of the antisera gave the results similar to that obtained with each single treatment. These findings showed that leptospiral attachment appears to be mediated by fibronectin, collagen and laminin.

To ascertain whether the extent of attachment correlate to the virulence potential, the leptospiral attachment tests to component-coated coverslips were carried out using lines of different virulence and the reference strains (avirulent) of the same serovars (Table 3). The highly virulent line of *copenhageni* attached to collagen more effectively than the two avirulent lines of the same strain or reference strain M20 (avirulent) ($P < 0.01$). In the case of *canicola* Moulton and *pomona* MLS, the number of attached leptospires of both highly virulent lines was higher than that of the intermediate virulent lines ($P < 0.01$). The number

Table 2. Effect of antifibronectin, anticollagen and antilaminin antibodies on leptospiral attachment to ECM^{a)}

Antibodies	Numbers of Attached Leptospires ^{b)} (Mean $\pm$ S.E.)	% of Inhibition
Normal Rabbit Serum	66.5 $\pm$ 3.9	0
Antifibronectin	45.7 $\pm$ 5.2	31.3
Anticollagen	44.9 $\pm$ 7.3	32.5
Antilaminin	47.7 $\pm$ 3.6	28.3
Antifibronectin, Anticollagen Antilaminin	28.7 $\pm$ 4.9	56.8

a) ECM was pretreated with 10 μ l antiserum or control serum for overnight at 4°C prior to co-incubation with *L. interrogans* serovar *copenhageni* Shibaura line No.1 for 2 hr at 30°C.

b) Per $\times 600$ (40 $\times$ 15) field. Twenty fields were examined.

Table 3. Attachment of highly virulent, intermediately virulent and avirulent lines of leptospira to four structural components of ECM^{a)}

Strains (Lines)	Virus- lence ^{b)}	Numbers of Attached Leptospires ^{c)} (Mean±S.E.) to			
		Collagen (400 µg/ml)	Fibronectin (400 µg/ml)	Laminin (400 µg/ml)	Hyaluronic Acid (2000 µg/ml)
<i>copenhageni</i>					
Shibaura					
(Line No.1)	HV	296.8±41.3	71.2±21.1	71.0±15.2	44.9±5.3
(Line No.3)	AV	41.4±19.7 ^{d)}	15.3± 4.9 ^{d)}	37.7± 5.6 ^{d)}	45.4±3.8
(Line No.5)	AV	58.9±16.4 ^{d)}	24.6± 5.7 ^{d)}	43.0± 8.1 ^{d)}	46.7±7.4
M 20	AV	12.1± 5.4 ^{d)}	30.1± 4.6 ^{d)}	32.9± 3.4 ^{d)}	39.5±6.0
<i>canicola</i>					
Moulton					
	HV	311.6±53.2	66.7±18.2	59.9±17.8	44.1±5.2
	IV	62.5±13.8 ^{d)}	27.4±11.2 ^{d)}	39.8± 9.1 ^{d)}	47.5±5.9
Hond Utrecht	AV	23.2± 7.2 ^{d)}	9.8± 3.3 ^{d)}	15.9± 4.5 ^{d)}	39.0±4.1
IV					
<i>pomona</i>					
MLS					
	HV	378.3±55.3	38.1± 7.5	75.2±10.8	35.6±3.8
	IV	50.9±10.6 ^{d)}	19.7± 4.8 ^{d)}	49.3± 5.5 ^{d)}	31.2±4.2
Pomona	AV	17.4± 3.5 ^{d)}	10.2± 2.3 ^{d)}	32.2± 3.1 ^{d)}	39.6±6.5

a) Co-incubation for 2hr at 30°C.

b) HV: highly virulent, IV: intermediately virulent, AV: avirulent.

c) Per ×600 (40×15) field. Twenty fields were examined.

d) P<0.01 when compared with each virulent strain.

of attached leptospires of each reference strain (avirulent) was less than that of both the highly and intermediately virulent lines ($P<0.01$). Similar findings were obtained in the adhesion of leptospires to fibronectin and laminin, but leptospiral attachment to hyaluronic acid did not correlate to virulence. Repeated experiments gave similar results.

DISCUSSION

The mechanisms of leptospiral attachment, which are considered to be an important first step in leptospiral infection, are not yet clear. Previous reports [1, 11, 21, 24] have shown that leptospires attached to several culture cells. We have previously shown that leptospires also attach to the ECM that exists between cells and tissues [9]. This paper demonstrated that leptospires attached to four structural components

of ECM, collagen, fibronectin, laminin and hyaluronic acid. Furthermore, the presence of soluble fibronectin, collagen and laminin, and the pretreatment of ECM with anti-fibronectin, anticollagen and antilaminin antisera decreased the number of attached leptospires to ECM. These findings may suggest that leptospires attach to host tissues through these components.

A previous report [6] showed that *Treponema pallidum* also attached to these four components. However, Thomas *et al.* [17] suggested that fibronectin was the principal mediator of *T. pallidum* cytoadherence because only antifibronectin serum diminished adherence of treponemes to cells. Our data indicated that preincubation of ECM with antiserum to fibronectin, collagen or laminin resulted in partial blockage (approximately 30%) of leptospiral attachment in any cases. Various experimental conditions were tested, but we were unsuccessful in

increasing the level of blockage with any serum. Furthermore, pretreatment of ECM with a mixture of three antisera did not show total blockage of attachment. Leptospire may attach to ECM through fibronectin, collagen and laminin, to a considerable extent, and the attachment may be completed by attachment through some unknown components.

Enhancement of leptospiral attachment to ECM was found in the presence of low concentrations (50–100 $\mu\text{g/ml}$) of soluble fibronectin and collagen. It is known that fibronectin has a specific binding site to collagen [5]. The enhancement of leptospiral attachment to ECM in the presence of soluble fibronectin and collagen may be based on the cross-binding that occurs between the ECM-associated fibronectin and collagen and the leptospira-associated ones. Inhibition of leptospiral attachment to ECM was found in the presence of a high concentration (400 $\mu\text{g/ml}$) of soluble fibronectin and collagen. This may be due to blockage of ligand by the soluble components. Furthermore, extra soluble components may also block the cross-binding sites of the ECM-associated fibronectin and collagen, which resulted in inhibition of leptospiral attachment to ECM.

RGDS tetrapeptide has been shown to be a target for treponemal attachment to fibronectin [19]. It would be interesting to know if a similar mechanism exists with leptospiral attachment to fibronectin. However, a synthetic RGDS tetrapeptide showed little or no inhibition of leptospiral attachment, suggesting that leptospire attach to a site of fibronectin different from RGDS tetrapeptide to which *T. pallidum* attach.

Fibronectin has heparin, fibrin and gelatin binding domains, in addition to cell binding domain containing RGDS [8]. *Staphylococcus aureus* bind to the NH_2 -terminal heparin and fibrin binding domain [12]. Coincuba-

tion of fibronectin with heparin, fibrin and gelatin did not affect leptospiral attachment to fibronectin (author's unpublished data). Although the study is still preliminary, the results might show that leptospire did not bind to any of heparin, fibrin and gelatin binding domains. Further investigations using the fragment of each domain of fibronectin are needed before determining the binding site of fibronectin to leptospire.

Leptospiral attachment to fibronectin, collagen and laminin was correlated to virulence, but no such correlation was seen with hyaluronic acid. It is possible that virulent leptospire possess ligands for fibronectin, collagen, laminin and hyaluronic acid, while avirulent leptospire lack the ligands for former three components but possess the ligand only for hyaluronic acid. These possibilities are subjects of future study.

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要 約

細胞外基質を構成する4つの成分へのレプトスピラの付着：伊藤寿啓・梁川 良（北海道大学獣医学部家畜衛生学講座）——細胞外基質構成4成分であるフィブロネクチン，コラーゲン，ラミニン及びヒアルロン酸に対するレプトスピラの付着を各々の成分塗布カバースリップを用いて検討した。生菌の付着数はいずれの成分に対しても各々その濃度に依存して増大したが，不活化菌はほとんど付着しなかった。またフィブロネクチン，コラーゲン及びラミニンへの付着菌数はヒアルロン酸への付着菌数に比べて有意に多かった。可溶性のフィブロネクチン，コラーゲン及びラミニン存在下では，レプトスピラの細胞外基質への付着は阻止されたが，ヒアルロン酸は阻止活性を示さなかった。細胞外基質を抗フィブロネクチン血清，抗コラーゲン血清あるいは抗ラミニン血清で前処理するとレプトスピラの付着菌数は減少した。レプトスピラ強毒クローンは弱毒クローンよりもフィブロネクチン，コラーゲン及びラミニンに有意に多く付着したが，ヒアルロン酸への付着は毒力の程度と関連しなかった。以上の結果から，*L. interrogans*はフィブロネクチン，コラーゲン及びラミニンを介して宿主組織へ付着することが示唆された。