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Restriction Endonuclease DNA Analysis of *Leptospira interrogans* Serovars *icterohaemorrhagiae* and *hebdomadis*

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Antigenic variants of *Leptospira interrogans* serovars *copenhageni* and *hebdomadis* were examined by bacterial restriction endonuclease DNA analysis with *Eco*RI, *Xho*I, *Sal*I, *Bst*EII, and *Hin*dIII as the digesting enzymes. The antigenic variants were stable cloned strains which had been cultivated in media containing homologous immune serum. One of the strains examined has been reported elsewhere (R. Yanagawa and J. Takashima, Infect. Immun. 10:1439–1442) as having an antigenic makeup which more closely resembles serovar *kremastos* than the serovar *hebdomadis* parent. The closely antigenically related but naturally occurring serovars *icterhaemorrhagiae* strain RGA and *copenhageni* strain M20 were examined in parallel. No differences could be shown between the *hebdomadis* parent and any of its mutants. Serovars *copenhageni* and *icterohaemorrhagiae* produced patterns which differed in the high-molecular-weight bands only. The Shibaura parent strain did not differ from *copenhageni* M20, but the Shibaura M1 strain differed from the other mutants and from *icterohaemorrhagiae* RGA in its high-molecular-weight bands.

If cloned leptospiral strains are cultivated in a medium which contains homologous immune serum, antigenic variants, both stable and unstable, may be selected (1, 2, 10, 11, 13, 15). The emergence of stable antigenic variants has been explained as being due to either one-step mutations or successive mutations (2, 10). The degree to which the selection of such antigenic variants occurs in nature has not yet been determined. It is thought by some researchers to offer a possible explanation for the in vivo selection of new leptospiral serovars (10, 15). Yanagawa and Takashima (17) claim to have converted *Leptospira interrogans* serovar *hebdomadis* to serovar *kremastos* by this method. These authors do not necessarily contend, however, that under natural conditions new serovars are emerging frequently due to this type of selection pressure. The antigenic makeup of different serovars within the definition as laid down by the Taxonomic Subcommittee in 1978 is considered to be remarkably stable (4).

This study was undertaken to look for differences between antigenic variants of *Leptospira interrogans* serovars *copenhageni* and *hebdomadis* generated during growth in homologous antiserum (15, 17), using bacterial restriction endonuclease DNA analysis (BRENDA). This technique has been undertaken on a large number of different members of the genus *Leptospira*, both type cultures and field isolates, with *Eco*RI as the digesting enzyme, and has been found to produce stable and reproducible patterns (8, 12). To see if the degree of difference between mutants is comparable to that observed between closely related naturally occurring organisms, we examined serovars *icterohaemorrhagiae* RGA and *copenhageni* M20 in parallel by the same method.

Bacterial strains. Strains M11, M12, M15, and 124-1, which are antigenic variants of *L. interrogans* serovar *copenhageni* Shibaura, were isolated by Yanagawa and Adachi (14). Serovars *icterohaemorrhagiae* RGA and *copenhageni* M20 were obtained from the World Health Organization Collaborating Center, Leptospirosis Reference Laboratory, Centers for Disease Control, Atlanta, Ga. The *L. interrogans* serovar *hebdomadis* parent strain and the derived mutant strains have been described previously (17). Two of these strains (strains 102 and 106) have been shown to have antigenic characteristics similar to those of serovar *kremastos* (17). All strains were grown in liquid EMJH medium (Difco Laboratories, Detroit, Mich.).

Preparation of DNA. Leptospiral DNA was extracted by the method described by Marshall et al. (8). After extraction, each sample was dialyzed exhaustively against TE buffer (10 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA) at 4°C. The absorbance (Unicam SP500 spectrophotometer) of each preparation was measured at 260 nm in a quartz-glass cell with a 1-cm light path; it was assumed that one optical density unit was equivalent to 50 μg of double-stranded DNA per ml. To calculate the contribution of RNA to the absorbance, the DNA content of each preparation was also measured by fluorimetry (7), using calf thymus DNA (type 1; Sigma Chemical Co., St. Louis, Mo.) to construct a standard curve of 0 to 1 μg of DNA per ml.

Restriction endonuclease digestion of DNA. Two micrograms of bacterial DNA was completely digested at 37°C for 1 h with 2 to 5 units of restriction endonucleases (*Eco*RI, *Xho*I, *Sal*I, *Bst*EII, *Hin*dIII; New England Biolabs, Beverly, Mass.) in the appropriate enzyme buffer made according to the manufacturer's specifications.

Gel electrophoresis and photography. Gel electrophoresis and photography were performed by the method of Marshall et al. (8), with the exception that electrophoresis was maintained at 5 V/cm until the tracking dye (bromophenol blue) had traveled a distance of 12 cm (ca. 3.5 h).

When DNAs extracted from the mutants M11, M12, M15, and 124-1 and the parent *L. interrogans* serovar *copenhageni* Shibaura were cut with *Eco*RI, *Hin*dIII, *Bst*EII, *Xho*I, and *Sal*I, no differences among mutants were seen in their respective electrophoretic patterns. Strain M1, however, produced some additional high-molecular-weight bands (Fig. 1). A series of dilutions of *Eco*RI were used for this particular strain, and the high-molecular-weight bands remained unaltered.

No differences could be shown between the serovar *hebdomadis* parent and mutants 101, 102, and 106 (Fig. 2)

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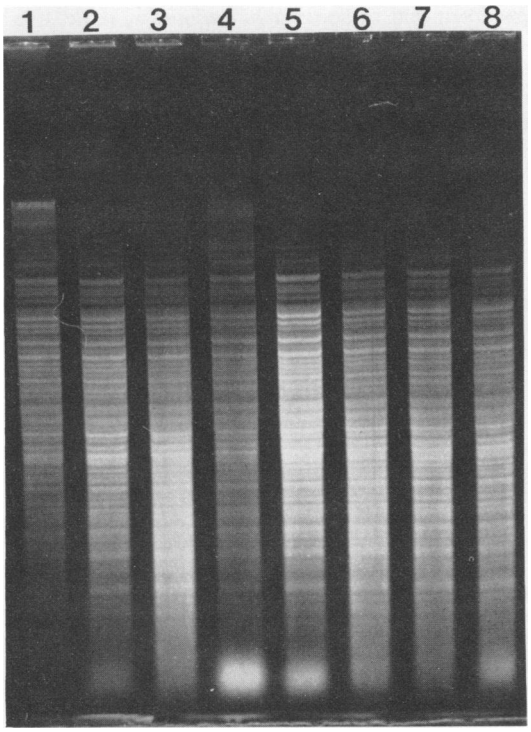

FIG. 1. *Eco*RI digest of DNA from *L. interrogans* serovar *icterohaemorrhagiae* RGA (lane 1), *copenhageni* M20 (lane 2), *copenhageni* Shibaura parent (lane 3), Shibaura M1 (lane 4), Shibaura M11 (lane 5), Shibaura M12 (lane 6), Shibaura M15 (lane 7), and Shibaura 124-1 (lane 8).

with any of the enzymes listed above. Serovars *copenhageni* M20 and *icterohaemorrhagiae* RGA produced patterns with *Eco*RI which differed in that RGA had some higher-molecular-weight bands not produced by M20 (Fig. 1). This difference was consistently present with a range of various concentrations of enzyme. Differences between the DNAs of these two *Leptospira* strains were also discernable with the enzymes *Hin*dIII, *Bst*EII, *Xho*I, and *Sal*I (data not shown).

Unlike the electrophoresis of proteins (polyacrylamide gel electrophoresis) which has been used for fingerprinting bacteria, the electrophoresis of bacterial DNA is not influenced by the type of environment in which the bacteria are grown. BRENDA should therefore be the ideal technique for investigating bacterial variants whose selection has come about as a result of alterations to their growth medium. However, some mutant strains of bacteria which have been investigated by restriction endonuclease DNA analysis cannot be differentiated (6, 9). Mielenz et al. (9) examined mutant strains of *Rhizobium japonicum* using *Eco*RI as their restricting enzyme, and they were unable to see any differences in DNA pattern despite obvious differences in colonial morphology. *Streptomyces glaucescens* mutants were examined by Hintermann et al. (6) using four different enzymes, and they concluded that except for a few specific variants the banding patterns between mutant strains remained unchanged. The exceptions were seen in strains derived as a result of exposure to mutagenic treatments such as growth in the presence of ethidium bromide. The *copenhageni* and *hebdomadis* organisms that were used in this present study had been grown in the presence of homologous immune serum, tested by agglutinin absorption and precipitin absorption, and shown to be antigenically different (15,17). The antigenic changes which were induced are in some cases of the order that had these variants been naturally occurring and stable they would be considered different serovars. However, when the BRENDA method was applied, no differences could be shown among the *hebdomadis* strains (Fig. 2), and in only one *copenhageni* strain (M1) did the DNA pattern show a difference (Fig. 1). When the M1 strain of *copenhageni* Shibaura was digested with other restriction enzymes (*Xho*I, *Sal*I, *Hin*dIII, and *Bst*EII) no distinction could be made between this strain, the parent strain, or any of the other mutants. This indicates that perhaps only very minor changes to the DNA had occurred and that in only one case did this change alter the cutting sites recognized by the enzymes used.

In 1938 Borg-Peterson (3) described two antigenic subtypes of serovar *icterohaemorrhagiae* represented by strains RGA and M20. As a result of his agglutinin absorption tests, he concluded that strain M20 possessed an antigenic component that was additional to those present in the RGA strain. He also concluded that both subtypes exist in nature and that the absence of some antigenic component in the RGA strain was not the result of prolonged cultivation in artificial medium. Each of these subtypes has since been given the status of serovar, and the M20 strain has been named *copenhageni*. It is interesting to note in these BRENDA studies that the close similarity between these two strains also extends to their restriction endonuclease DNA analysis patterns. The patterns bear a much greater resemblance to one another than do those of any other two serovars of *Leptospira* studied by this method. When digested with *Eco*RI, the serovars are easily distinguished from one another, with a number of differences throughout the length of the patterns. The presence of distinctive high-molecular-weight bands in the RGA strain which are not seen in the M20 gels is the only feature that makes them distinguishable. Different dilutions of restriction enzyme were used with the strain

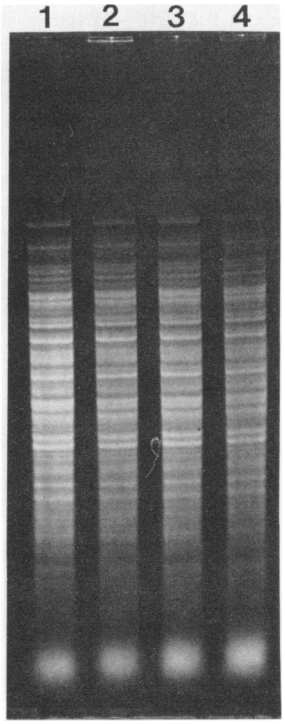

FIG. 2. *Eco*RI digest of DNA from *L. interrogans* serovar *hebdomadis* parent (lane 1), mutant 101 (lane 2), mutant 102 (lane 3), and mutant 106 (lane 4).

RGA and M20 preparations to ensure that these rather poorly resolved high-molecular-weight bands had not resulted from partial digestion of the DNA. The positions of the high-molecular-weight bands of RGA are similar to, but not identical with, the bands appearing in the *copenhageni* Shibaura M1 strain. Different patterns were generated when *icterohaemorrhagiae* RGA and *copenhageni* M20 were digested with each of the other enzymes used in this study, enabling them to be easily differentiated from one another. The inability of BRENDA to differentiate among some mutant strains of *Leptospira* does not place any practical limitation on its usefulness as a technique for serovar identification or for epidemiological studies. The mutants, if those are what they are, which it fails to identify have been created as a result of in vitro manipulation. Other similar antigenic variants have been observed in leptospires cultured from the blood from experimentally infected mice, guinea pigs, puppies, and pigs (5, 15, 16). However, the stability of these in nature has not been tested, nor have these variants been shown to be shed in the urine. In order for antigenic variants to be perpetuated it is necessary for them to be shed in the urine of naturally infected animals. Whether these antigenic variations and even the variations in colony size described by Yanagawa et al. (16) are due to changes in the DNA molecule has not been conclusively demonstrated.

In the case of strain *copenhageni* Shibaura M1 in which DNA changes are detected, they have only been seen with *Eco*RI digests and are therefore not comparable in degree to the differences in the DNA fingerprint among different serovars. It is possible that changes in the base sequence of the DNA molecule can occur in sections of the DNA between cutting sites and thereby remain undetected by BRENDA. Nonstable variants may remain viable when cultured in an artificial medium, and these variants may also occur in nature but may be selected against because of their inability to be shed in the urine of the host.

The report by Yanagawa and Takashima (17) that a cloned culture of serovar *hebdomadis* had been changed to *kremastos* has particularly important epidemiological implications. A culture of this antigenically changed organism was represented in this study by strains 102 and 106. When seeking an explanation for this phenomenon consideration must be given to the possibility that the original culture was a mixture of the two serovars. If the majority of organisms in the original culture were serovar *hebdomadis* but were mixed with an undetectably low level of *kremastos*, then growth in anti-*hebdomadis* antiserum would bring about an apparent change in the identity of the culture by allowing the *kremastos* element of the culture to flourish. This explanation, however, has been discounted because the original inoculation strain was cloned and this was followed by a long period of laboratory subculturing. Despite the microscopic agglutination absorption and immunodiffusion evidence that *hebdomadis* mutants 102 and 106 were antigenically similar to *kremastos*, their BRENDA patterns when cut with *Eco*RI, *Hin*dIII, *Bst*EII, *Xho*I, and *Sal*I show them still to be identical to both the *hebdomadis* parent and mutant 101. The hypothesis that a change to another serovar can result from the selection of antigenic variants which are induced by exposure to immune serum is not upheld by this study. The BRENDA pattern produced by serovar *kremastos* is very different from that of serovar *hebdomadis*, indicating that a substantial rearrangement of DNA is required to bring about a true conversion of *hebdomadis* to *kremastos*. There is no indication that any such rearrangement has occurred after the growth of *hebdomadis* organisms in the presence of homologous immune serum.

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