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SUBSURFACE LOCATION OF NON-AGGLUTINATING ANTIGENS OF *LEPTOSPIRA INTERROGANS* (SEROTYPE *BIFLEXA*)

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Summary. Neither monoprecipitin rabbit antiserum against precipitating antigen 'd' nor 'e' of *Leptospira interrogans* (serotype *biflexa*) would agglutinate whole leptospires, even at very low antiserum dilution.

By means of antibody fluorescent labelling and anti-globulin cross-linking techniques, antigen 'd' and 'e' were shown to be subsurface in cellular location.

INTRODUCTION.

In a previous paper Graves and Faine (1974) showed that a trypsin extract of *Leptospira interrogans* (serotype *biflexa*) contained precipitating antigens designated 'axial filament' (Chang and Faine, 1970), 'd' and 'e', when immunodiffused against a rabbit antileptospiral serum. Monospecific antisera against both antigen 'd' and antigen 'e' have been prepared.

In this paper the role of antigens 'd' and 'e' in the agglutination of leptospires and their cellular location was investigated. Most other antigen systems of leptospires described previously were involved in leptospiral agglutination by specific or group antisera.

The role of individual antigens in the agglutination of leptospires has obvious significance for understanding leptospiral structure, in immunity to infection and in diagnosis.

MATERIALS AND METHODS.

Methods for the cultivation of leptospires, preparation of trypsin extract (TE) and antigen 'd' and the production of monoprecipitin antisera against both antigen 'd' and 'e' were described previously (Graves and Faine, 1974). Goat anti-rabbit serum was purchased from Pentex (Miles Laboratories, U.S.A.).

Fluorescein labelling of rabbit antileptospiral sera.

(a) *Preparation of the globulin.* The globulin was precipitated at 40% saturation with ammonium sulphate. After standing for 1 h the globulin was centrifuged at 28,000 *g* for 10 min, suspended in 1-2 ml water and dialysed overnight against 0·01M phosphate buffered saline, pH 7·2 (PBS).

(b) *Conjugation of globulin to fluorescein isothiocyanate, isomer* 1: (*FITC*). The method of The and Feltkamp (1970) was used. The labelled globulin pool was concentrated by negative pressure dialysis, then dialysed overnight against PBS. The concentrated conjugate was sterilized by filtration through a cellulose acetate membrane filter (APD, 0·45μ) and stored at 4°.

Fluorescein-labelled goat anti-rabbit serum was purchased from Wellcome (Burroughs-Wellcome, Australia).

Reaction of leptospires with fluorescein-labelled antisera.

(a) *Direct fluorescent antibody test with alcohol fixed leptospires.* After drying in the air, the leptospires were fixed by immersing the glass slide in ethanol for 15 sec. The slides were then dried at 37°.

The fluorescein-labelled rabbit antiserum, e.g. rabbit anti-'d' serum either diluted or undiluted, was added to the leptospires on the slide and allowed to react for 30 min at room temperature in a humid atmosphere. The antiserum was then poured off and the slide washed with agitation for about 1 h in 1 ml of PBS and blotted dry. The preparation was mounted in buffered glycerol (9 parts glycerol to 1 part veronal buffered saline pH 8·6).

(b) *Direct fluorescent antibody test with unfixed leptospires.* In a typical procedure 1 drop of a washed culture of leptospires was mixed with 2-3 drops of fluorescein-labelled rabbit antiserum, in a 15 ml glass centrifuge tube and allowed to react for 1 h at room temperature without drying out. The mixture was then washed at least three times in volumes of 5 ml veronal buffered saline pH 7·2. After the final centrifugation the sedimented leptospires were suspended in 2 or 3 drops of buffer. A loopful was placed on an acid-washed glass slide, allowed to dry in air and mounted in buffered glycerol.

Sensitization of whole leptospires with rabbit antileptospiral sera and subsequent cross-linking with goat anti-rabbit serum.

(i) *Preliminary titrations.*

(a) Titration of rabbit antileptospiral serum. This was a standard microscopic agglutination test (WHO, 1967).

(b) Titration of a formalized leptospiral suspension. A suitable concentration of formalized leptospires was found which did not completely agglutinate in the presence of 1/500 diluted rabbit anti-'whole leptospire' serum, as follows. Formalin to a final concentration of 0·2% was added to a 5 day culture of leptospires in rabbit serum Korthof medium, which was then shaken and left for at least 2 h before use. Serial two-fold dilutions of this leptospiral suspension were prepared and to each dilution was added an equal volume of a 1/500 dilution of rabbit anti-'whole leptospire' serum, previously heated at 56° for 30 min. A 1/20 dilution of leptospiral suspension was found to be suitable for subsequent use.

(c) Titration of goat anti-rabbit serum. Two sets of serial two-fold dilutions of goat anti-rabbit serum, heated at 56° for 30 min were prepared from 1/10 to 1/10,240 in veronal buffered saline, pH 7·2. To each tube of one set of dilutions was added an equal volume of a 1/20 dilution of formalized leptospires. A 1/20 dilution of formalized leptospires previously treated with 1/500 rabbit anti-'whole leptospire' serum was added to the other set. In this way the optimal dilution of goat anti-rabbit serum for differentiating between 'uncoated' and 'antibody-coated' formalized leptospires was determined.

(ii) *Attempted cross-linking of antibody-coated leptospires with antiglobulin.*

Four suspensions of leptospires, A, B, C and D were prepared, each with different antibody treatment as follows.

Suspension A. Uncoated leptospires prepared by mixing 0·5 ml of a 1/20 diluted formalized leptospiral suspension from a 5 day culture with 0·5 ml veronal buffered saline pH 7·2.

Suspension C. Antibody-coated leptospires prepared by mixing 0·5 ml of a 1/20 dilution of formalized leptospiral suspension from a 5 day culture with 0·5 ml antileptospiral serum of appropriate specificity, e.g. anti-'d', which had previously been heated at 56° for 30 min to destroy complement.

Suspensions A and C were each incubated for 1 h at 30° with occasional shaking, after which suspensions B and D were prepared.

Suspension B. To 0·5 ml A was added 0·5 ml of a 1/2,000 dilution of goat anti-rabbit serum previously heated at 56° for 30 min.

Suspension D. To 0·5 ml C was added 0·5 ml of the same preparation of a 1/2,000 dilution of heated goat anti-rabbit serum as in B. Suspensions B and D were then each incubated separately for 1 h at 30° with occasional shaking. At the end of this period, counts of free, unagglutinated leptospires were made of suspensions A, B, C (controls) and D (test), in a Petroff-Hauser counting chamber. At least 400 leptospires from several samples were counted from each mixture. The counting procedure took from 4 to 6 h, during which time the cultures stood at room temperature. Samples for counting were taken in rotation from the four reaction mixtures. The different antisera used to 'coat' the formalized leptospires in suspension C were as follows:

(i) rabbit anti-'whole leptospire' serum, 1/500 and 1/1,000 dilutions,
(ii) normal rabbit serum, 1/10 and 1/500 dilutions,
(iii) rabbit anti-'d' serum, 1/10 and 1/100 dilutions, and
(iv) rabbit anti-'e' serum, 1/10 and 1/40 dilutions.

Fluorescent microscopy.

A Zeiss microscope was used in conjugation with a quartz-iodine lamp and a Zeiss KP 500 exciter filter and barrier filter No. 50 (transmission wave lengths 500 nm and over). A x 25 objective (N.A. = 0·45) was used with a x 10 eyepiece and a 'Tiyoda' oil immersion darkfield condenser (N.A. 1·2-1·4).

RESULTS.

Agglutinating activity of the monoprecipitin antisera.

All anti-'e' and anti-'d' sera, whether produced from purified column fractions, immunoadsorbent eluents or immune precipitates in agar (Graves and Faine, 1974), consistently failed to agglutinate whole leptospires in a standard leptospiral microscopic agglutination test (WHO, 1967).

Immunofluorescence of leptospires with monospecific antisera.

The four fluorescein-labelled antisera used in this study were normal rabbit globulin; rabbit anti-'whole leptospire' globulin; rabbit anti-'d' globulin; and rabbit anti-'e' globulin. Each individual serum was added directly to the leptospires. The indirect fluorescent antibody technique was used initially but was abandoned because of the nonspecific fluorescence of leptospires pre-treated with normal rabbit serum.

(a) *Direct immunofluorescence of alcohol fixed leptospires.* Anti-'whole leptospire', anti-'d' and anti-'e' sera all reacted with whole, fixed leptospires although normal rabbit globulin did not react.

Trypsinized leptospires reacted less strongly with anti-'d' than did whole leptospires. A preparation of purified antigen 'd', left to dry on a glass slide, and allowed to react with anti-'d' showed specific fluorescence on the spot where the solution had been placed, although no structures were apparent. In both cases where the trypsinized leptospires fluoresced, the leptospiral debris released by trypsin (Graves and Faine, 1974) also fluoresced, in some cases apparently more strongly than the remaining leptospires.

To check the specificity of the reactions, each antiserum was allowed to react at various dilutions with alcohol fixed preparations of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Streptococcus pyogenes.* A non specific reaction occurred between all the sera including normal rabbit globulin and *Staph. aureus*, but there was no reaction between the other bacteria and the fluorescein conjugated globulins at the dilutions used to stain the leptospires.

(b) *Direct immunofluorescence of unfixed leptospires.* Irrespective of whether a low or high ratio of antiserum to leptospires was used, neither anti-'d', anti-'e', nor normal rabbit globulin would stain the leptospires. In contrast, anti-'whole leptospire' globulin did stain the leptospires. The same staining pattern was observed with trypsinized leptospires, presumably because antigens 'd' and 'e' had been removed from the cells by the trypsin and released into solution.

Sensitization of whole leptospires with anti-'d' and anti-'e' serum for anti-globulin cross-linking.

Three different experimental procedures were used in an attempt to detect rabbit antibodies present on the surface of leptospires after their treatment with anti-'d' or anti-'e' sera.

1. An anti-globulin procedure used with a 1/20 dilution of either goat anti-rabbit serum or anti-rabbit globulin failed to agglutinate leptospires to a higher titre than the 2^{11} reached with antileptospiral serum alone.

2. Leptospires grown in sheep-serum medium were treated with rabbit antisera to whole leptospires, or antigens 'd' or 'e'. The suspensions were sonicated and rabbit globulin sought by diffusing the sonicated suspension against goat anti-rabbit serum. No precipitin lines were seen.

3. Rabbit antileptospiral antibodies were detected on the surface of leptospires pre-treated with rabbit antileptospiral serum by cross-linking with goat anti-rabbit serum. The anti-globulin method (1 above) was modified using leptospires more heavily coated with antibody, but with a much lower concentration of leptospires than in a standard M.A.T. Cross-linking occurred with leptospires pre-treated with rabbit anti-'whole leptospire' serum but not with leptospires pre-treated with normal rabbit serum, or rabbit anti-'d' serum or rabbit anti-'e' serum (Table 1).

A statistical analysis of the results indicated that only by precoating the leptospires with anti-'whole leptospire' serum was it possible to further enhance agglutination by the addition of goat anti-rabbit serum (1/2,000 dilution). The differences due to the other three precoating sera were not significant at the 0·95 level.

TABLE 1.

Effect of a 1 *in* 2000 *solution of goat anti-rabbit serum on leptospires pre-treated with various rabbit sera.*

Rabbit serum used for pre-treatment (column 1)	Reciprocal-dilution	Percentage reduction in concentration of unagglutinated leptospires following goat anti-rabbit serum after pre-treatment with		
		nothing	rabbit serum (see column 1)	difference due to rabbit serum
normal	500	38·6	39·6	+1·0
	10	49·4	39·7	−9·7
anti-whole leptospire	1000	42·0	68·8	+26·8*
	500	32·5	56·3	+23·8*
anti-'d'	100	45·8	44·0	−1·8
	10	47·2	46·8	−0·4
anti-'e'	40	29·9	39·4	+9·5
	10	33·5	41·8	+8·3

* Differences significant at 0·95 level.

DISCUSSION.

Neither anti-'d' nor anti-'e' sera had detectable leptospiral agglutinating ability. This could be explained by two hypotheses:

(i) Antigens 'd' and 'e' are not agglutinating antigens because they are located below the leptospiral surface. Their site either prevents access of antibodies to the antigen in the whole leptospire or prevents cross-linking because the antibody, when attached to the antigen, is too far into the cell to make contact with another leptospiral cell.

(ii) Some of the anti-'d' and anti-'e' antibodies are monovalent, blocking antibodies which preferentially combine with the antigenic sites, thereby preventing the multivalent antibodies from combining with the sites and cross-linking the cells. It is not known whether monovalent antibodies were present, but multivalent antibodies were clearly present because the sera formed strong precipitin bands in immunodiffusions.

The finding that the two antigens 'd' and 'e' are definitely not involved in leptospiral whole cell agglutination was unexpected. In this respect, antigens 'd' and 'e' fit Rothstein and Hiatt's (1956) hypothetical 'S' class of somatic antigens, not located peripherally, which do not take part in whole cell agglutination. However, when the cell is disrupted they react with their homologous antibodies; for example, in immunodiffusion.

Leptospiral antigen preparations prepared by other workers have usually given rise in rabbits to antibodies which agglutinate leptospires ('agglutinating antibodies') (Schneider, 1955; Cox *et al.*, 1958; Schricker and Hanson, 1963; Faine, Adler and Ruta, 1974). On the other hand, leptospiral lipids (Stalheim, 1968) did not induce agglutinins although they fixed complement in the presence of antisera. It is likely that these lipids act as haptens and thus do not precipitate

with homologous antisera. In contrast, antigens 'd' and 'e' precipitated with antisera which did not agglutinate the whole leptospires.

The strong cross-reaction between *Staph. aureus* and all the 4 fluorescein-conjugated antisera, including normal rabbit globulin, was probably due to staphylococcal antigen, 'Protein A', which reacts with normal sera from a variety of animals (Lind *et al.*, 1970; Franek *et al.*, 1971). Pepsin digestion of immunoglobulin preparations (Forsgren and Forsum, 1970) eliminated this reaction, showing that the Fc fragments of the antibody were involved. The reaction is therefore not a true antigen-antibody reaction.

The cross-linking of antibody-coated leptospires with goat anti-rabbit serum was demonstrated, once the correct leptospiral concentration and anti-rabbit serum concentration to detect the cross-linking had been established. Ford and De Falco (1956) increased the titre of a number of bacterial agglutinating sera by adding anti-globulin to the washed cells in each antiserum dilution tube. In most cases it was an increase of one doubling dilution in titre.

In this study it was not possible to enhance the titre of a leptospiral agglutination titration in this manner. Only by counting free unagglutinated leptospires before and after treatment with anti-globulin with the necessary controls was it possible to detect cross-linking. Leptospires treated with anti-'d' or anti-'e' serum, even at relatively high concentrations, could not be cross-linked with anti-rabbit serum. This is clear evidence that antigens 'd' and 'e' are not available as surface antigens but are possibly located deeper in the cell, certainly too far in for anti-rabbit serum antibodies to cross-link adjacent leptospires.

Indeed, there is no good evidence that anti-'d' and anti-'e' antibodies react with intact leptospires. If the antigens are indeed subsurface, such a reaction would depend on the permeability of the outer layers of the leptospiral cell to antibody protein molecules and the depth in the cell at which these antigens are localized. It is certain, however, that damage to the cell, as by trypsinization, releases or exposes an antigen. Alcohol fixation may render the cell permeable to fluorescein-labelled antibodies against antigens 'd' and 'e' or allow their exposure following removal of the envelope layer (Yanagawa and Faine, 1966). In contrast, normal leptospiral cells do not release antigens 'd' and 'e' since culture supernatants contain no detectable 'd' or 'e' (Graves and Faine, 1974).

Furthermore, fluorescein-labelled anti-'e' and anti-'d' did not attach to free leptospires, possibly because of lack of access of antibody to antigen (van Driel *et al.*, 1973).

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