The detection of leptospires by a chemiluminescent immunoassay

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Summary. Rabbit serum hyperimmune to *Leptospira interrogans* var *icterohaemorrhagiae* serovar *icterohaemorrhagiae*, reference strain RGA was conjugated to the chemiluminescent label ABEI (6-[N-(4-aminobutyl)-N-ethyl amino]-2,3-dihydrophthalazine-1,4-dione) in the presence of the coupling agent, EDC (1-ethyl-3-(3-dimethyl amino-propyl)-carbodiimide HCl). The luminescent conjugate was incubated with homologous and heterologous antigens. The results indicate that chemiluminescence may provide an accurate and rapid method of detecting leptospires in biological fluids.

Introduction

Leptospirosis is probably the most widespread zoonosis in the world (van der Hoeden, 1964). The predominant infecting serogroups found in Great Britain are *Leptospira interrogans* var *icterohaemorrhagiae* (the causative agent of Weil's disease), var *canicola* (causing jaundice in dogs and man) and var *hebdomadis*, particularly serovar *hardjo* (causing mastitis and abortion in cows and also human infection) (Waitkins, 1983). In 1983, 45% of all human leptospiral infections in the British Isles were caused by serovar *hardjo*; the majority of patients were cowmen or farmers who regularly milked their cows in “herringbone” milking parlours. In veterinary practice, it has been estimated that 30% of all abortions in cattle are associated with serovar *hardjo* and one third of all cattle herds in England and Wales are infected with this leptospire (Ellis et al., 1982a and b).

Laboratory diagnosis of human and animal leptospirosis can be difficult. Serovar *hardjo* may be isolated from cow urine by use of complex selective and enriched media with incubation for several weeks. Leptospirosis may be diagnosed in aborted fetuses by examining tissue specimens by the fluorescent-labelled antibody technique (Smith et al., 1967). Both isolation and fluorescent methods depend upon viable leptospires surviving in specimens which often arrive in the laboratory autolysed or are delayed in transit. Serological evidence of leptospiral infections in animals may be sought but the interpretation of results can be difficult, particularly in an older cow which has aborted several weeks before being tested.

There are similar but more acute problems in the laboratory diagnosis of human leptospirosis. The specialised media necessary for successful isolation of leptospires from biological fluids are not readily available in routine primary-care hospital laboratories. Specimen collection also requires specialised methods; for example, human urine is slightly acid and therefore kills freshly voided leptospires. Patients should raise the pH of their urine before it is examined for leptospires by taking potassium citrate. There are similar problems in the investigation of blood cultures and cerebro-spinal fluid. Furthermore, if leptospires are found they must be cultured and identified before their clinical significance can be established. These procedures may take several weeks or months.

The laboratory diagnosis of human leptospirosis depends almost exclusively on serological methods, and almost always proves to be retrospective. A rapid, reliable and easy laboratory test which would establish a diagnosis of leptospirosis at the acute clinical stage of the disease would be an invaluable aid to clinicians. No such test is yet available either in human or veterinary practice. The use of chemiluminescence for detecting leptospires in biological fluid is a novel and, we believe, unique approach to the detection of any bacteria, not only leptospires.

Materials and methods

Preparation of antiserum

One-ml volumes of Freund's complete adjuvant containing c. (1 x 10⁸)-(1 x 10⁹) cells of *L. interrogans* var *icterohaemorrhagiae* serovar *icterohaemorrhagiae*, reference strain RGA were injected subcutaneously into rabbits. Inoculations were repeated at weekly intervals for 5 weeks, when 10–20 ml of blood was collected from the ear vein. The hyperimmune serum was separated by
centrifugation at 2700 g and stored at −30 °C until required. The protein concentration of the serum was estimated with the biuret-phenol reagent (Sigma Chemicals Co., Poole, Dorset) and the anti-leptospire serological activity determined by the microscopic agglutination test (see below).

Chemiluminescent conjugation of antiserum

The rabbit anti-leptospire serum was diluted with 0.1 N HCl to a concentration of protein c. 10 mg/ml. The method for conjugation of antiserum followed that of Hømmila and Lövgren (1982). Briefly, 1 ml of serum was first mixed with 200 μl of ABEI (6-[N-(4-aminobutyl)-N-ethyl amino]-2,3-dihydrophthalazine-1,4-dione) (Sigma). The ABEI was prepared as a 50 mM stock solution by dissolving the powder in 0.1 N HCl. Solid EDC (1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide HCl) (Sigma) 3.5 mg was then added at hourly intervals. The coupling took place at 37°C for 3 h and excess reagent was removed by gel filtration.

The conjugate (200 μl) was loaded on to the surface of a Sephadex G-50 (medium grade; Pharmacia Fine Chemicals, Pharmacia Limited, Milton Keynes) column (20 cm × 16 mm internal diameter) and eluted with 0.001 N HCl, pH 3.0 at a flow rate of 15.1 ml/cm²/h. The absorbance of the effluent was monitored at 280 nm (LKB Ultrospec 4050; LKB Instruments Limited, Croydon, Surrey) and 2-ml fractions were collected (LKB Fraction Collector 2112). Those fractions containing labelled protein and exhibiting luminescent activity were stored at −30 °C until required for testing.

The protein concentration of the conjugate was estimated with the biuret-phenol reagent (Sigma) and the serological activity by the microscopic agglutination test.

Preparation of conjugated antiserum–antigen complex

The antigens were L. interrogans var icterohaemorrhagiae serovar icterohaemorrhagiae, reference strain RGA, L. interrogans var canicola serovar canicola, reference strain Hond Utrecht IV, and L. interrogans var hebdomadis serovar hebdomadis, reference strain hebdomadis. Each was subcultured aseptically into 20 ml of sterile EMJH broth (Ellinghausen and McCullough, 1965; Johnson and Harris, 1967) and incubated at 37°C for 7-10 days. The purity of the cultures was checked by dark-field microscopy and 250-μl samples, containing c. (1 × 10^9)–(1 × 10^10) cells/ml, were aseptically pipetted into sterile 5-ml screw-capped bottles.

Conjugated serum (250 μl) was added to each antigen preparation. The suspensions were mixed gently, incubated at 37°C for 2 h and centrifuged at 27 000 g for 10 min. The supernates (50-μl samples) and pellets (10 μl) were then assayed in triplicate for chemiluminescent activity.

Chemiluminescent assay

The reaction mixture for the chemiluminescent assay (Hømmila and Lövgren 1982) contained: 50 mM phosphate buffer, pH 8.6; 0.5 ml; Microperoxidase (LKB; diluted 1 in 10^3 in distilled water) 50 μl; 10 mM H₂O₂ 0.45 ml; Sample 50 μl unless otherwise specified. The assay was initiated by injecting H₂O₂ into the reactant solution. Luminescence was measured with a 1250 Luminometer (LKB) connected to a 1223 Luminescence Analyzer (LKB). The integrated signal was measured for 10 s (T1–10 mv. s).

Microscopic agglutination test

The microscopic agglutination test was modified from Schüffner and Mochtar (1927). Unconjugated and conjugated serum was diluted in phosphate-buffered saline, pH 7.0 (Oxoid) to final doubling dilutions of 1 in 51 200 and 1 in 128, respectively, and 50 μl-volumes of each dilution were pipetted into wells of a WHO perspex tray. Antigen preparations grown in EMJH broth (Ellinghausen and McCullough, 1965; Johnson and Harris, 1967) for 7–10 days at 30°C were checked for purity by dark-field microscopy and 50 μl were added to each well of the WHO perspex tray containing serum or conjugate. The WHO perspex tray was placed in a moist chamber and incubated at 37°C for 2 h. The degrees of agglutination were determined by dark-field microscopy at a magnification of × 400.

Results

The biological and chemical properties of the rabbit anti-leptospire (L. interrogans var icterohaemorrhagiae) serum before and after conjugation to ABEI are given in table I. The protein concentration of the conjugate after gel filtration was 0.2808 mg/ml and the microscopic agglutination test end-point titre with homologous antigen was c. 100. The chemiluminescent activity of the conjugate was 134-5 (T1–10 mv. s).

The chemiluminescent activity of each antigen is shown in table II. The properties of the conjugate in reactions with each antigen are shown in table III. There was no microscopic agglutination in conjugate-heterologous antigen reactions (L. interrogans var Canicola and var hebdomadis), and the luminescent activities of the supernates and pellets after centrifugation were approximately 35 and 7 T1–100 mv. s, respectively. In contrast, in conjugate-homologous antigen reactions (L. interrogans var icterohaemorrhagiae) the end-point microscopic agglutination titre was 2–4 and the luminescent activities of the supernate and pellet after centrifugation were 32-00 and 41-14 T1–10 mv. s, respectively.

Discussion

The serological activity of the hyperimmune
CHEMILUMINESCENT DETECTION OF LEPTOSPIRES

Table I. The properties of hyperimmune serum before and after conjugation to ABEI*

<table>
<thead>
<tr>
<th>Serum before conjugation</th>
<th>Serum after conjugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein concentration (mg/ml)</td>
<td>Micro-agglutination end-point titre</td>
</tr>
<tr>
<td>9.83</td>
<td>1000</td>
</tr>
</tbody>
</table>

* 6-[N-(4-aminobutyl)-N-ethyl amino]-2,3-dihydrophthalazine-1,4-dione.

Table II. Chemiluminescent activity of antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Luminescence in T1-10 mv. s</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. interrogans var canicola, serovar canicola, Hond Utrecht IV</td>
<td>0.03</td>
</tr>
<tr>
<td>L. interrogans var hebdomadis, serovar hebdomadis, Hebdomadis</td>
<td>0.07</td>
</tr>
<tr>
<td>L. interrogans var icterohaemorrhagiae, serovar icterohaemorrhagiae, RGA</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table III. Properties of antigen-conjugate reactions

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Antigen</th>
<th>Antigen-conjugate reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Micro-agglutination end-point titre</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. interrogans, var icterohaemorrhagiae, serovar icterohaemorrhagiae, RGA; ABEI labelled</td>
<td>L. interrogans var canicola, serovar canicola, Hond Utrecht IV</td>
<td>0</td>
</tr>
<tr>
<td>L. interrogans var hebdomadis, serovar hebdomadis, Hebdomadis</td>
<td>0</td>
<td>35:75</td>
</tr>
<tr>
<td>L. interrogans var icterohaemorrhagiae, serovar icterohaemorrhagiae, RGA</td>
<td>2-4</td>
<td>32:00</td>
</tr>
</tbody>
</table>

serum was unaffected by labelling with ABEI (table I). The conjugate showed luminescent activity (134.5 T1-10 mv. s; table I) and had an A323/A280 ratio of 0.258 (table I). A similar absorbance ratio was reported by Hemmilä and Lövgren (1982) for ABEI-labelled rabbit anti-human IgG and indicates a high ratio of label to immunoglobulin.

The conjugate showed no serological activity in reactions with heterologous antigens (L. interrogans var canicola, serovar canicola, Hond Utrecht IV and L. interrogans var hebdomadis serovar hebdomadis, Hebdomadis) (table III). However, luminescent activity was detected in both supernate and pellet (table III) but this was due solely to the presence of free conjugate.

With homologous antigen (L. interrogans var icterohaemorrhagiae serovar icterohaemorrhagiae, RGA) the conjugated serum showed serological activity (table III), and luminescent activity was detected in both supernate (32-00 T1-10 mv. s, table III) and pellet (41-14 T1-10 mv. s, table III) after centrifugation. These results may indicate that some of the labelled immunoglobulins in the complex hyperimmune serum were non-specific. Thus, the presence of both non-specific and specific antibodies to the homologous antigen would account for the observed luminescent activities in the supernate and pellet, respectively (table III). However, the activity of the centrifuged pellet, which also gave agglutination visible by dark-field microscopy, was much higher than that found with heterologous antigens (table III) and indicates that leptospires were detected in vitro. These results are encouraging, though further studies are required. It is likely that
greater sensitivity and specificity of the conjugate-antigen reaction would be gained by the purification of IgG immunoglobulin and by the use of monoclonal antibodies against specific leptospiral serovars. The luminescent activity of ABEI is lowered by conjugation to immunoglobulin and sensitivity would be improved by using other chemiluminescent agents. This is currently being studied.

In conclusion, the preliminary results outlined in this report indicate that the chemiluminescent technique should be further studied and may replace current methods for the detection of leptospire infection.

REFERENCES


