Effect of Heat or Formalin Treatment of Leptospires on Antibody Response Detected by Immunoblotting

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Leptospira interrogans serovar icterohaemorrhagiae RGA (RGA), live or heated at 56°C for 15 min or treated with Formalin, was injected into rabbits to prepare hyperimmune serum. The pathogens L. interrogans serovars icterohaemorrhagiae RGA, icterohaemorrhagiae 1, canicola Moulton, grippotyphosa Andaman, hardjo Hardjoprajito, and pomona Pomona and the nonpathogen Leptospira biflexa serovar Patoc 1 were processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and after electrophoresis they were then transferred to nitrocellulose paper. Antiserum against RGA (live, heat killed, or Formalin killed) was used on one of each of the three blots. Formalin appeared to completely eliminate antibody response to antigens with the molecular weight of 14,000 to 20,000 (14K to 20K) but did expose an antigen at approximately 23K in the pathogens only. This same band had only slight reactivity when antiserum against heat-killed RGA was used. Heating also eliminated cross-reactivity in the 19K to 30K range and partially degraded bands in the 14K to 20K region so that one broad band rather than several discrete bands appeared. The three antiserum specimens cross-reacted with all of the serovars tested, but few antigens of grippotyphosa and hardjo reacted with the antiserum. Against patoc, reactivity was limited primarily to the flagellar region. The most cross-reactivity was with the antiserum prepared by using live leptospires.

Control of leptospirosis in domestic animals, primarily dogs and livestock, involves the use of bacterins. Various methods of inactivating the spirochete have all resulted in some degree of failure in preventing renal infection or death (1-3, 8, 9, 12). We recently showed that heat or chemical treatment altered some antigens so that they were not detectable by immunoblotting with antiserum prepared against live organisms (14). It has been demonstrated that some treatments also reduced the titers obtained in the microscopic agglutination test (MAT) (11, 13, 14) as well as increased nonspecific reactivity (13). To the best of our knowledge, no studies have been done to indicate the effect of heat or Formalin treatment of leptospires on antibody formation as detected by Western immunoblots.

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To examine this question, we took a culture of Leptospira interrogans serovar icterohaemorrhagiae RGA (RGA) and produced hyperimmune serum (15) in three rabbits. Serum from the rabbits was pooled after checking the titers by the MAT (15). Three rabbits each were also inoculated with cultures either killed at 56°C for 15 min (RGA-H) or killed with 0.02% (final concentration) Formalin (RGA-F). The antiserum were tested in the MAT (15) against each of the serovars run in the blots. The results were presented in Table 1.

Cells (5 x 10⁸) (6) of the pathogenic serovars icterohaemorrhagiae RGA and 1, canicola Moulton, grippotyphosa Andaman, hardjo Hardjoprajitos, and pomona Pomona and the nonpathogen patoc Patoc I were centrifuged at 16,000 x g for 2 min, washed twice in 0.01 M phosphate-buffered saline, pH 7.2, and solubilized in 100 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. The samples were heated at 100°C for 10 min the day before electrophoresis was done and were stored at 4°C overnight. The next day, samples were run in duplicate on each of two 10% polyacrylamide gels according to the method of Laemmli (10), with the formulations of Hames (7) as previously described (13). Molecular-weight standards (Pharmacia, Inc., Piscataway, N.J.) were also included on each gel.

After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose (0.45-μm pore size), on the basis of the method of Towbin et al. (16), with 20% methanol in the Tris-glycine buffer. Transfer was done at 200 V for 2 h. The nitrocellulose sheets were then cut in half lengthwise. One section was stained with 0.2 g of amido black in 90 ml of methanol and 90 ml of deionized water to determine transfer efficiency. The three remaining sections were placed in separate containers and blocked with 2% normal rabbit serum in Tris-buffered saline with 0.5% gelatin and 0.1% Tween 20. The three anti-RGA antiserum specimens were diluted 1:100 in Tris-buffered saline and incubated on the blots, with agitation, at room temperature for 2 h. The horseradish peroxidase-conjugated anti-rabbit immunoglobulin (ICN ImmunoBiologicals, Lisle, Ill.) was diluted 1:2,000 in Tris-buffered saline with 0.1% Tween 20 according to the manufacturer’s directions and incubated on an orbital shaker at room temperature for 2 h. A solution of 25 mg of diamino benzidine (Sigma Chemical Co., St. Louis, Mo.) and 30 μl of 3% H₂O₂ (Mallickrodt, Inc., Paris, Ky.) in 50 ml of 1 x Tris-buffered saline was used for a substrate. Blots were then rinsed in several changes of deionized water to stop the reaction.

The three antiserum specimens reacted with bands of about 67,000 (67K), 30K to 43K, and 14.4K (Fig. 1). The most obvious change was the reduction of cross-reactivity...
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<td></td>
<td>RGA</td>
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<td>RGA</td>
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<td>Icterohaemorrhagiae 1</td>
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<td>Canicola</td>
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<td>Grippotyphosa</td>
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With canicola and pomona when killed leptospires were used to produce antiserum. Antiserum produced against RGA reacted with distinct bands of about 14.4K, but these bands appeared as a diffuse wide band when antiserum against RGA-H was used. These bands did not react at all with antiserum to RGA-F, indicating alteration by Formalin. Although there was less cross-reactivity with the antiserum against RGA-F, a band that was not present on the RGA blot and was only vaguely visible with RGA-H appeared quite strongly at approximately 23K. The band at about 40K was the most intense with this same antiserum. The most intense reaction with the three antiserum specimens was with bands in the 34K to 37K range. None of the bands below 30K, including the 14.4K- and 20.1K-molecular-weight markers, showed up on the amido black-stained nitrocellulose (data not shown).

The bands that appeared in the 14K to 16K range are most likely leptospiral lipopolysaccharide (LPS) (5), while those at 34K to 37K are probably flagellar proteins (4). Spirochetal flagella are located between the protoplasmic cylinder and the outer envelope, which might partially explain why the 34K to 37K bands remained equal in intensity regardless of treatment to the leptospires. It is also apparent that heat or Formalin treatment alters the LPS enough that its antigenicity is either altered or eliminated. This could be important, since results by Cinco et al. (5) suggest that the LPS contains the epitope responsible for serovar-specific reactions. Degradation of the LPS would allow other antigenic sites to be exposed, which might account for the appearance of the band at about 23K with RGA-F antibody. Loss of the LPS and the uncovering of the flagellar proteins, which appear to be genus specific (4), might explain the broader cross-reactivity in the MAT. Formalin treatment of the antigen also caused a stronger antibody reaction with the 41K to 42K band and greater cross-reactivity in the MAT.

Studies by Palmer et al. (13) indicated that Formalin-treated antigens frequently had lower titers when homologous antisera were tested but had broader reactivity and were thus able to detect antibodies when the infecting serovar was not used and MAT antigens were negative. Heating at 56°C also has been shown to decrease agglutination titers (11, 14), although not to the same extent as Formalin treatment. Although we did not find any significant reduction in titers, our results suggest that changes in titers and increased nonspecificity may be due in part to alteration of the LPS, since either heat or Formalin treatment of the spirochete appears to affect the immune response to bands in this area, as detected by immunoblotting. This would suggest that caution must be taken in pretreating leptospires before injection into animals for production of antigens, since pretreatment may alter the immune response to the desired antigens. Pretreatment may also enhance antibody response, as indicated by the increased response to the 41K to 42K band. This may be important when trying to produce a monoclonal antibody against such a band.

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REFERENCES


