Serological Follow-Up of Patients Involved in a Localized Outbreak of Leptospirosis

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Eighteen patients involved in a localized outbreak of leptospirosis were subjected to a serological follow-up study over a 5-year period. Four distinct sets of sera from all patients and a fifth sample obtained from 10 of them were examined by the microscopic agglutination test (MAT) for demonstration of leptospiral antibodies. The test was carried out by using live leptospires from reference strains of 17 Leptospira interrogans serovars known to occur in Italy. In all cases, the highest titers of agglutinins were recorded against one or more of the three Australis group serovars tested (australis, bratislava, and lora). The highest antibody levels were reached soon after the acute phase of infection in some patients but only after some months in others. Titters then tended to recede with varying rapidity, but titters against the Australis group serovars were still detectable in some patients after 5 years. Coagglutinins against serovars of other serogroups were detected, generally at low levels, in the early sets of sera of most patients, but tended to disappear in the late-set sera. Specific immunoglobulin M (IgM) and IgG against the three Australis group serovars were determined in most serum samples from 16 patients by solid-phase enzyme immunoassay (EIA). In general, EIA titters were considerably lower than MAT titters, but there was a certain patient-to-patient variability in both the IgM/IgG ratio and the evolution and persistence of the two immunoglobulin classes. Since all the evidence indicated that the initial outbreak was from a single source, the observed patient-to-patient variability in the progress of both MAT and EIA titters appeared to be attributable to factors inherent in the individual patients. Cross agglutination absorption tests, aimed at retrospectively determining to which of the Australis group serovars the outbreak-specific infecting strain belonged, were performed with six serum samples from different patients. Most absorbed sera seemed to originate from an australis or lora infection, but it was not possible to discriminate conclusively between the two serovars.

In Italy, until the 1960s, leptospirosis was a relatively common occupational infection, especially in rice field workers (4). Subsequently, with the use of prophylactic measures and the mechanization of rice cultivation, the morbidity rate decreased and a different risk group pattern more related to recreational activities began to emerge. Recent studies have shown a marked decrease in the Bataviae serogroup infections (formerly predominating in rice field workers (4)), a relative persistence of infections caused by the Icterohaemorrhagiae group serovars, and the emergence of serovars of the Javanica and Australis serogroups (7, 9, 10).

The present study shows the results of a serological 5-year follow-up of patients involved in a localized, waterborne outbreak of leptospirosis that occurred during the summer of 1984 in a small town of central Italy. As reported previously (5), serologically confirmed cases occurring during the outbreak totalled 33, with a fatality rate of 8.6%; epidemiologic study showed that the patients contracted the infection by drinking water from a fountain contaminated by a hedgehog trapped in an adjoining reservoir. Considering that no Leptospira strain could be isolated during the outbreak, we also attempted to investigate the identity of the outbreak-specific infecting serovar retrospectively.

**MATERIALS AND METHODS**

**Patients and sera.** Of the 33 cases of serologically confirmed leptospirosis which occurred during the outbreak (5), we succeeded in monitoring 18 patients (15 males and 3 females, ranging in age from 35 to 78 years at the time of the outbreak) over a 5-year period. All had had clinical manifestations of the disease during the outbreak. Four serum samples were obtained from each of the 18 patients, while a fifth sample was obtained from 10. A serum sample collected during or immediately after the epidemic outbreak, i.e., by the third or fourth week after the onset of disease, was considered to be the first sample. The second to fifth samples were obtained approximately 9, 18, 36, and 54 months after the outbreak.

**Agglutination tests.** The microscopic agglutination test (MAT) was used as the reference method for the demonstration of leptospiral antibodies (3, 11). The assay was carried out by using live leptospires from reference strains of 17 Leptospira interrogans serovars known to occur in Italy; reference strains from 4 serovars of L. biflexa were also included in the battery (Table 1). The strains were cultivated and counted as described previously (8). Each serum sample was tested at a screening dilution of 1:50. Positive sera were titrated by the interlocking scheme, by using dilutions of 1:100, 1:320, 1:1,000, 1:3,200, 1:10,000, and 1:32,000. Microscopic readings were performed by using an agglutination endpoint of 50%. A positive control serum with a known titer and a negative control of diluent alone were always included.

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Cross agglutination absorption tests were employed for retrospective investigation. Suspensions (0.05%) of 10^10 formalized leptospires of reference strains of the Australis group serovars present in Italy were used as the absorbing antigens. Selected test sera were absorbed with these suspensions at a dilution of 1:25. After overnight incubation at 37°C, residual postabsorption titers were detected by the MAT with both the homologous and heterologous strains, according to Dikken and Kmetý’s procedure (11).

Solid-phase EIA. Specific immunoglobulin M (IgM) and IgG were determined by enzyme immunoassay (EIA). Leptospires of strain Riccio 2 (serovar bratislava) were centrifuged at 10,000 × g for 15 min and suspended in 0.06 M carbonate buffer (pH 9.6) to a concentration of 5 × 10^6/ml. They were sonicated at 20 kHz for two periods of 15 s each. Flat-bottom microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 µl of the sonicated suspension. The wells were washed three times with phosphate-buffered saline (PBS) and then coated with 1% (wt/vol) bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) at room temperature for 30 min. After three washings with PBS as described above, twofold dilutions of the serum sample starting with 1:40 were added, and the plates were incubated at 37°C for 1 h. After three washings with PBS containing 0.05% (wt/vol) Tween 20, 100 µl of antihuman IgM or IgG alkaline phosphatase conjugate (Sigma) diluted 1:1,000 was added to each well, and the microplate was incubated for 1 h at 37°C. Finally, the wells were washed three times with PBS-Tween 20 and supplemented with 100 µl of a 1-mg/ml solution of p-nitrophenylphosphate. After 30 min of incubation at 37°C, the enzyme substrate reaction was stopped by adding 50 µl of 1 M NaOH, and the optical density at 450 nm of the solution was read with a Unicam II spectrophotometer (Labsystems Oy, Helsinki, Finland). Each specimen was tested in duplicate. Substrate and conjugate controls were included in each plate, as well as negative and positive control sera.

The cutoff point was previously determined on 20 sera (diluted 1:40) from healthy blood donors. The mean optical densities of all negative sera for the anti-IgM and anti-IgG conjugates were calculated as 0.116 and 0.101, respectively. The values corresponding to the means plus 3 standard deviations were taken as the lowest levels at which specific IgM and IgG were considered to be present. Results were expressed as titers, corresponding to the dilutions at which an optical density greater than or equal to the cutoff was recorded.

RESULTS

In all of the 18 cases followed up, the highest titers of agglutinins yielded by the MAT were recorded against one or more of the Australis group serovars. In particular, the highest levels of agglutinins were reached against the serovar lora in six patients; against lora and bratislava in four; against australis, lora, and bratislava in three; against bratislava in three; and against australis in two. The titer curves obtained for the three serovars are shown in Fig. 1. In general, despite a certain variability in absolute values, high-titer antibodies against one or more of the three serovars were detected in the early-set sera (first or second sample). However, the evolution of the titers over the approximately 9 months that elapsed between the first and the second sample varied considerably from patient to patient as well as with the serovar tested. Some agglutinin titers were found to rise in some patients while remaining unchanged or decreasing in others. No further increase in titers was observed after another 9 months (third sample): agglutinins against any given serovar either stayed at the same level or decreased in all patients. A tendency toward a general decrease in titers was noted in the sera obtained 3 years or more after the outbreak. In the last set of sera (which included samples available from only 10 of the 18 patients), only 1 case showed agglutinins exceeding 1:100, and in 5 cases, agglutinins became undetectable (<1:50) against all serovars.

Coantibodies (i.e., nonspecific cross agglutinins) against serovars of other serogroups were detected in 14 of the 18 sera collected soon after the acute phase of infection. These coagglutinins were more often directed towards the serovars copenhageni, zanoni, poi, and sejroe. The titers were generally low (in only one patient was a value of 1:1,000 reached [against the serovar zanoni]) and tended to recede in the succeeding samples, with virtually no coantibody being detectable 3 years later.

Specific IgM and IgG were determined in most serum samples from 16 patients, by using strain Riccio 2 (serovar bratislava) as the antigen. EIA titers were in general considerably lower compared with the MAT titers yielded by the same samples against the Australis group serovars (Table 2). A long persistence of IgM was noted in some cases, with titers sometimes higher than those of IgG. However, there was a certain patient-to-patient variability in both the IgM/ IgG ratio and the evolution and persistence of the two immunoglobulin classes.

Cross agglutination absorption tests were performed with six serum samples from different patients cross-absorbed with reference strains of the three Australis group serovars. However, considering that the infecting serovar should absorb homologous agglutinins and that 10% or more of the homologous titer should remain after absorption with heterologous antigens (15), the data obtained for the retrospective determination of the identity of the outbreak-specific serovar were not conclusive. The results obtained with serum 17b suggested australis, those with three other sera (1b, 4a, and 14b) suggested australis or lora, and those with another sample (3a) suggested australis or bratislava as the serovar.

**TABLE 1. Live leptospires used for the MAT**

<table>
<thead>
<tr>
<th>Species</th>
<th>Serogroup</th>
<th>Serovar</th>
<th>Strain</th>
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<tbody>
<tr>
<td><em>L. interrogans</em></td>
<td>Australis</td>
<td>Ballico</td>
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<td></td>
<td>Bratislava</td>
<td>Riccio 2</td>
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<td></td>
<td>Lora</td>
<td>Riccio 37</td>
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<td></td>
<td>Bataviae</td>
<td>Castellonis</td>
<td>Castellon 3</td>
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<td>Canicola</td>
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<td>Grippotyphosa</td>
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<td></td>
<td>Hebdomadis</td>
<td>Mini</td>
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<td></td>
<td>Icterohaemorragiae</td>
<td>Icterohaemorragiae</td>
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<tr>
<td></td>
<td>Javanica</td>
<td>Poi</td>
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<td></td>
<td>Pomona</td>
<td>Pomona</td>
<td>Mezzano 1</td>
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<td></td>
<td>Pyrogenes</td>
<td>Zanoni</td>
<td>Zanoni</td>
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<td>Sejroe</td>
<td>Hardjo</td>
<td>Hardjoprajitno</td>
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<td>Saxkoebing</td>
<td>Mus 24</td>
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<td>Tarassovi</td>
<td>Tarassovi</td>
<td>Mitis Johnson</td>
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<td>Andama</td>
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<td>CH 11</td>
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<td>Patoc</td>
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<td>Sao paulo</td>
<td>Sao Paulo</td>
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</table>

*Note:* The MAT titers were determined by EIA with specific antibodies against *L. interrogans* serovars. The cutoff point was determined as 1:40 against healthy blood donors.
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responsible for the infection; with the sixth sample (6b), no residual titer was obtained after absorption with any of the three Australis group serovars (Table 3).

DISCUSSION

Human leptospirosis is generally sporadic and only rarely occurs in outbreaks. Agglutinins usually become detectable by the end of the first week of disease and reach maximal levels by the third or fourth week. Thereafter, antibodies gradually decline but may be detectable for months and even years, with a titer curve which may vary with the interplay of factors inherent in the infecting leptospiral strain and factors inherent in the individual host (3, 14). The present serological follow-up study of patients involved in a localized outbreak of leptospirosis substantially confirms that agglutinins may persist for years. However, it even more clearly confirms that individual factors may strongly influence the progress of the agglutinating antibody response. In fact, with the initial outbreak appearing from all evidence to be from a single source, the same strain was the common infecting agent in all patients monitored, who had apparently contracted the infection by drinking water from a contaminated fountain (5). Therefore, factors inherent in the individual patients are virtually the only means of accounting for the observed variability in the titer curves, with the highest titers recorded against different Australis group serovars and reached early in some patients but only after some months in others and then receding with a variably rapid trend.

Patient-to-patient variability was also observed when specific IgM and IgG were determined by EIA, which, unlike MAT, detects antibodies against many different leptospiral antigens, not necessarily only those involved in agglutination (2). In fact, besides the surface antigens (involved in MAT), immunogenic components of the cell wall, flagellin, and protoplasm also contribute to EIA reactivity. On the other hand, unlike in animal infections, the antibody response in human leptospirosis does not seem to follow the classical IgM and IgG pattern: the unusually long persistence of antileptospiral IgM agglutinins and the different abilities of individual patients to produce IgG agglutinins are well documented (1, 6). The fact that, in general, EIA titers were considerably lower than MAT titers is not surprising in a follow-up study, since EIA is an essentially genus-specific test (12) particularly sensitive in acutely infected patients (13).

It is generally accepted that, in human leptospirosis, serological tests may provide clues to the identity of the infecting serovar, but it may be difficult to draw firm conclusions unless the causative strain has been isolated and typed. However, although it was not possible to culture any Leptospira strain from the patients involved in the outbreak, both the epidemiologic investigation carried out soon after

FIG. 1. Titer curves obtained against three Australis group serovars, australis (■), bratislava (▲), and lora (●), by using the MAT in 18 patients who were monitored. Each patient number is reported in the top right corner of each box, with the sex (M, male; F, female) and age (years, referring to the time of the outbreak) reported below. The first serum sample (a) was collected during or immediately after the epidemic outbreak; the second to fifth samples (b to e) were obtained approximately 9, 18, 36, and 54 months after the outbreak. The sera were tested at dilutions of 1:50, 1:100, 1:320, 1:1,000, 1:3,200, 1:10,000, and 1:32,000. Titers are expressed as the log10 of the reciprocal.

[Diagram showing titer curves for different patients and serovars]
the outbreak (5) and the present serological follow-up study agree in indicating a strain of the serogroup Australis as the causative agent. The highest agglutinin titers were consistently recorded against the serovars of this serogroup, with coagglutinins virtually disappearing in the late-set sera, when the serovar immune response tends to become more specific.

Cross agglutination absorption tests proved to be of limited use in retrospectively determining to which of the Australis group serovars the outbreak-specific infecting strain belonged. The majority of the patient sera absorbed seemed to originate from an australis or lora infection, but a definitive discrimination between the two serovars could not be achieved. On the other hand, distinguishing between certain serovars of the serogroup Australis may be hard, even if polyclonal rabbit immune sera or monoclonal antibodies are used (9). The prevalence in the patient sera of common immunodominant fractions recognizing the australis and lora serovars, and less so the bratislava serovar, is in keeping with the subgrouping scheme proposed by Dikken and Kmety (11) in which, on the basis of factor analysis, australis and lora belong to the subgroup Australis and bratislava belongs to the subgroup Jalna. The prevalent reactivities toward serovars australis and lora were confirmed by preliminary results obtained by examining the test sera by using Western blot (immunoblot) analysis (data not shown).

ACKNOWLEDGMENTS

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