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Immunoblot Studies to Analyze Antibody to the *Rickettsia typhi* Group Antigen in Sera from Patients with Acute Febrile Cerebrovasculitis

K. E. HECHEMY,[1]* J. A. FOX,[1] D. H. M. GRÖSCHEL,[2] F. G. HAYDEN,[2] AND R. P. WENZEL[3]

Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201-0509[1]; Departments of Pathology and Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908[2]; and Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, Iowa 52242[3]

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In 1986, an unusual syndrome of acute febrile cerebrovasculitis in the Piedmont Region of Virginia was reported. All patients had encephalopathy and prior exposure to both a sylvan environment and flea-infested animals. The initial serological studies suggested a rickettsial origin, corroborating clinical, epidemiological, and histopathological findings. Sera from four of five patients were subsequently studied by immunoblotting. Unabsorbed and absorbed sera were tested with electrophoresed and electroblotted *Rickettsia typhi*, *Legionella bozemanii*, and *Proteus vulgaris* OX19 antigens. The unabsorbed sera reacted with all three antigens. The *P. vulgaris*- and *L. bozemanii*-absorbed sera reacted with *R. typhi* only and without significantly less intensity. In contrast, the reactivity of *R. typhi*-absorbed sera was significantly lower with all three antigens. These results indicate that these patients had specific antibodies to a typhus group antigen. Although our findings suggest that a rickettsia of the typhus group may have caused this syndrome, no definitive diagnosis could be achieved because a rickettsial organism was not isolated.

In 1986, Wenzel et al. described a new syndrome of acute cerebrovasculitis in five patients from Virginia (21). The patients ranged in age from 16 to 59 years and became ill in November and December 1983. Two patients died, a third was left with serious neurological sequelae, and the youngest two recovered. The clinical and epidemiological findings were suggestive of a rickettsial etiology. All patients had recent exposure to forests or wood and contact with flea-infested animals. Clinicopathological findings revealed (i) cerebrovasculitis and perivasculitis involving mostly venules and capillaries in brain biopsies; (ii) neurological findings in all four patients suggestive of multifocal cortical involvement; (iii) transient oculomotor signs or symptoms in the patients, underscoring the severity of brain stem and midbrain complications; and (iv) elevated alveolar-arterial pO_2 gradients, despite normal chest radiographs.

Although an etiological agent was not isolated by culturing or demonstrated by immunofluorescence studies performed on punch biopsies (19), serological studies on specimens from four patients revealed antibody responses to typhus group antigens in the indirect hemagglutination and latex agglutination tests and in the microimmunofluorescence test with an anti-human immunoglobulin M (IgM) fluorescein isothiocyanate conjugate (21). The antibody titers obtained in the three serological tests were borderline positive. In addition, ehrlichiosis, a newly recognized rickettsial disease (5, 14), was excluded by indirect fluorescent-antibody tests. Findings obtained with other serological tests were summarized previously (21).

One explanation for the antibody findings was that the low titers might have represented "background" levels among persons in areas endemic for rickettsial diseases (6). Furthermore, the antigen fraction used in the latex agglutination and indirect hemagglutination tests is presumed to be a lipopolysaccharide and/or carbohydrate moiety (10, 16) and could have epitopes in common with several gram-negative bacteria. Thus, the question arose as to whether specific background—or cross-reacting—antibodies might have been detected and led to a possible misinterpretation of the laboratory data.

We have examined the specificity of the antibodies to the typhus group antigens observed in immunoblot studies. The antigens used were *Rickettsia typhi* as a representative of the typhus group; *Legionella bozemanii* as an organism that has recently been shown to contain antigens which cross-react with antibodies from patients diagnosed serologically as having typhus (17); and *Proteus vulgaris* OX19, one of the antigens used in the Weil-Felix test (20). Although a rickettsial agent was not isolated, our results suggest that a typhuslike agent cannot be ruled out as a candidate to explain this syndrome.

MATERIALS AND METHODS

Sera. The sera used in this study were test sera that were collected serially from the four patients (patients 2 to 5) with cerebrovasculitis during hospitalization and that were stored at −70°C (21). Serum from patient 1 was not available because the patient died 1 day after admission to the hospital. The following control sera were used: (i) 18 randomly selected serum specimens collected from inpatients at the University of Virginia Hospital and submitted to the laboratory for nonsyphilis serodiagnostic testing (nontyphus patients); (ii) strongly positive and weakly positive control serum specimens collected from two typhus patients (used for the enzyme-linked immunosorbent assay [ELISA]); (iii) *R. typhi*-reactive control serum (RCT) used for immunoblot studies and obtained by pooling the serum specimens from six typhus patients with microimmunofluorescence titers of

* Corresponding author.

≥2,048; (iv) 57 serum specimens collected from patients serodiagnosed as having typhus group fever (3 were from patients in New York, 3 were from patients in North Carolina, and 51 were from patients in Texas); and (v) rabbit antisera to *L. bozemanii* and *P. vulgaris* OX19 prepared (8) for use as reactive test control sera because specific human antisera were not available for these antigens.

Antigens. The following antigens were used: *R. typhi* complement-fixing antigen with an antigen titer of ≥16 (Wilmington strain, obtained from Theodore Tzianabos, Centers for Disease Control); *P. vulgaris* OX19 (11) (obtained from Rocky Mountain Laboratories and maintained in Albany, N.Y., since 1960); and *L. bozemanii* ATCC 33217 (maintained in charcoal-yeast extract agar) (4). The nonrickettsial antigens were lyophilized and suspended to a concentration of 1 mg (dry weight) per ml for use.

ELISA. The ELISAs for the detection of antibodies to *R. typhi* and *L. bozemanii* were performed (18) as follows. Twenty-five microliters of 1:20-diluted *R. typhi* antigen or 25 μl (2 μg) of *L. bozemanii* antigen in sodium carbonate buffer (pH 9.6) was added to a microtiter plate. These antigen concentrations were found to be optimal by block titration. The antigens were force precipitated (9) at 5°C with 42% ethanol contraining 0.5% sodium acetate (wt/vol). On the next day, the ethanol-acetate solution was aspirated and the wells were washed with phosphate-buffered saline (pH 7.4) containing 0.1% Tween 20 (PBS-T). The uncoated sites of the wells were blocked by incubation with 125 μl of 3% bovine albumin for 30 min at 25°C. Fifty microliters of serially diluted serum in PBS-T (starting dilution, 1:25 or 1:40) was added to the wells and incubated at 37°C for 2 h, and the wells were washed with PBS-T three times and incubated at 37°C for 30 min with horseradish peroxidase–anti-human IgA + IgG + IgM (heavy and light chains) conjugate (HRP–anti-human immunoglobulin [Ig]). The wells were washed with PBS-T, and the antibodies were detected with 0.01% *o*-phenylenediamine–0.03% H_2O_2. The reaction was read with a model MR700 microplate reader (Dynatech Laboratories, Chantilly, Va.). The titer was the reciprocal of the highest serum dilution which had an optical density of 0.2 to 0.3.

Serum absorption. Seventy-five microliters of serum was mixed with 375 μl of saline and 375 μl of *R. typhi* antigen. Absorption with the nonrickettsial antigens was performed with an antigen concentration of 5 mg/ml of saline and with a concentration of 2.5 mg/ml each for the *P. vulgaris* and *L. bozemanii* antigens for combined absorption with both antigens. All incubations were performed at 37°C for 24 h. The mixtures were centrifuged, and the supernatants were filtered through 0.2-μm-pore-size filters.

Electrophoresis, electroblotting, and immunoblotting. The rickettsial antigens were solubilized in modified Laemmli buffer (12) at 37°C for 5 min and stored overnight at 5°C. The nonrickettsial antigens were solubilized by boiling of a suspension of antigens in Laemmli buffer in a water bath at 100°C for 5 min and stored overnight at 5°C. These conditions were found to be best for obtaining optimum banding patterns and were necessary for solubilizing the nonrickettsial antigens. Milder solubilization conditions did not result in banding of the antigens but resulted in a smear in the area of the higher-molecular-weight region. Ten to 40 μl of solubilized antigen per lane was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12). Electroblotting and immunoblotting were performed as described previously (10). HRP–anti-human Ig, HRP–anti-human IgG (γ chain specific), and HRP–anti-human IgM (μ chain specific) were used to detect antibodies. The enzyme substrate was 4-chloro-1-naphthol.

TABLE 1. ELISA titers in sera from the four patients for *R. typhi* and *L. bozemanii* antigens

Serum	Days after onset	Titer for:	
		R. typhi	*L. bozemanii*
Patient 2	12	160	320
	14	80	320
	16	160	320
	25	20	80
Patient 3	11	160	160
	16	160	160
	27	80	160
	37	40	80
	39	20	40
Patient 4	6	40	80
	11	40	80
	22	160	640
Patient 5	19	80	80
Eighteen randomly selected patients	NA[a]	≤40	≤40
Typhus-reactive controls			
Weakly positive		320	160
Strongly positive		10,240	2,560

[a] NA, not available.

RESULTS

ELISA. Sera from patients with cerebrovasculitis had borderline positive ELISA antibody titers with *R. typhi* (Table 1). These results corroborate the previously obtained microimmunofluorescence, indirect hemagglutination, and latex agglutination test results (21). Moreover, a change in titers (twofold or more) between sera collected early and late in the disease was observed in all three patients from whom several sera were still available. The titers decreased in the sera of patients 2 and 3 and increased in the serum of patient 4. Patient sera and RCT cross-reacted with the *L. bozemanii* antigen with titers that were within 1 dilution of those obtained with the *R. typhi* antigen.

The 57 control serum specimens from patients serodiagnosed as having typhus fever group antigen in the states of Texas, North Carolina, and New York were also tested concurrently by ELISA with the *R. typhi* or the *L. bozemanii* antigen. The cross-reactivity of these serum specimens with *L. bozemanii* was extensive both qualitatively (all serum specimens cross-reacted) and quantitatively (the ratios of titers [number of serum specimens] obtained with *R. typhi* to those obtained with *L. bozemanii* were 0.25 [1], 0.5 [4], 1 [9], 2 [10], 4 [15], and >4 [18]).

Time course of reactivity with typhus fever group antigen. Although immunoblotting is mostly a qualitative tool, changes in antigen band intensities may reflect changes in antibody levels during the course of the disease, provided that all sera from a given patient are tested at the same dilution. HRP–anti-human Ig was used to identify antibodies to *R. typhi* (Fig. 1 and 2). With serum from patient 2, the

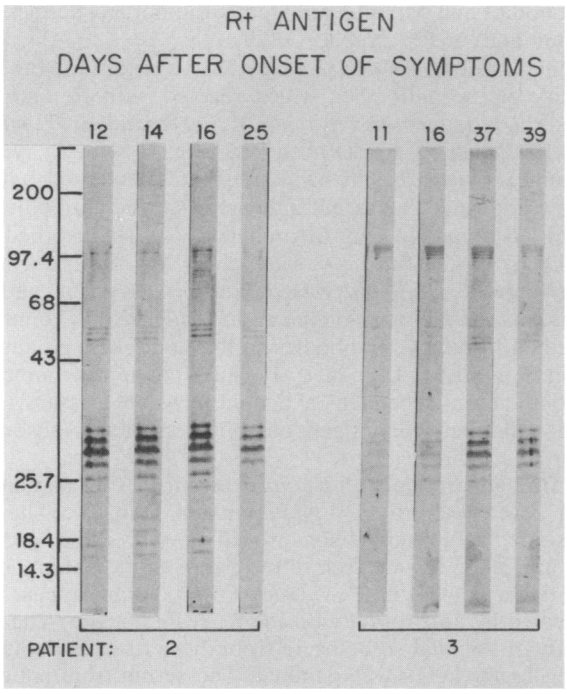

FIG. 1. Immunoblot profile of reactivity of antibodies to *R. typhi* (Rt) in sera obtained at different days after the onset of symptoms for patients 2 and 3. Numbers at left are in kilodaltons.

largest number of bands and maximum staining occurred on day 16 after onset. In addition, there was an apparent increase in the number of bands between 68 and 97.4 kDa. Serum collected at day 25 showed a significant decrease in the number of bands and the intensity of staining (Fig. 1). For patient 3, there was a significant increase in band intensity with sera collected on days 16 and 37. Compared with RCT and with sera from patients 4 and 5 (Fig. 2), sera from patients 2 and 3 had antibodies to a limited number of antigen bands, and the overall staining of the bands was subjectively considered to be of medium intensity. For patient 4, the intensity and the apparent number of bands detected peaked between days 13 and 22 (Fig. 2). The intensity of the bands decreased in the serum collected on day 31. Patient 5 exhibited a similar pattern, with maximum intensity observed in sera collected between days 23 and 29.

Cross-reactions. The immunoblot of each patient's serum with the highest ELISA titers to *R. typhi* is shown in Fig. 3. Patient sera and RCT appeared to have antibodies to both nonrickettsial organisms (Fig. 3). Antibodies to *L. bozemanii* were more prominent in all sera than antibodies to *P. vulgaris* OX19 and sometimes more prominent than those to the "homologous" *R. typhi* antigen. Additional immunoblot studies revealed that three serum specimens from the group of 18 random control serum specimens (nontyphus patients) had some degree of reactivity with at least one of the three antigens (Fig. 4, buffer panel for each serum specimen).

Ig class. To determine the Ig class reacting with the antigens, we selected the same sera as those used for the cross-reactivity study and RCT. RCT contained IgG and IgM to all three antigens (Fig. 5). However, the overall reactivity of IgM was more pronounced than that of IgG. Sera from three of the four patients (patients 2, 3, and 4) (Fig. 5) did not appear to have IgG antibodies to *R. typhi*. The serum from patient 5 contained a minimal level of IgG antibodies which reacted with the rickettsial antigen. In contrast, all patient sera had IgM antibodies to *R. typhi*. Sera

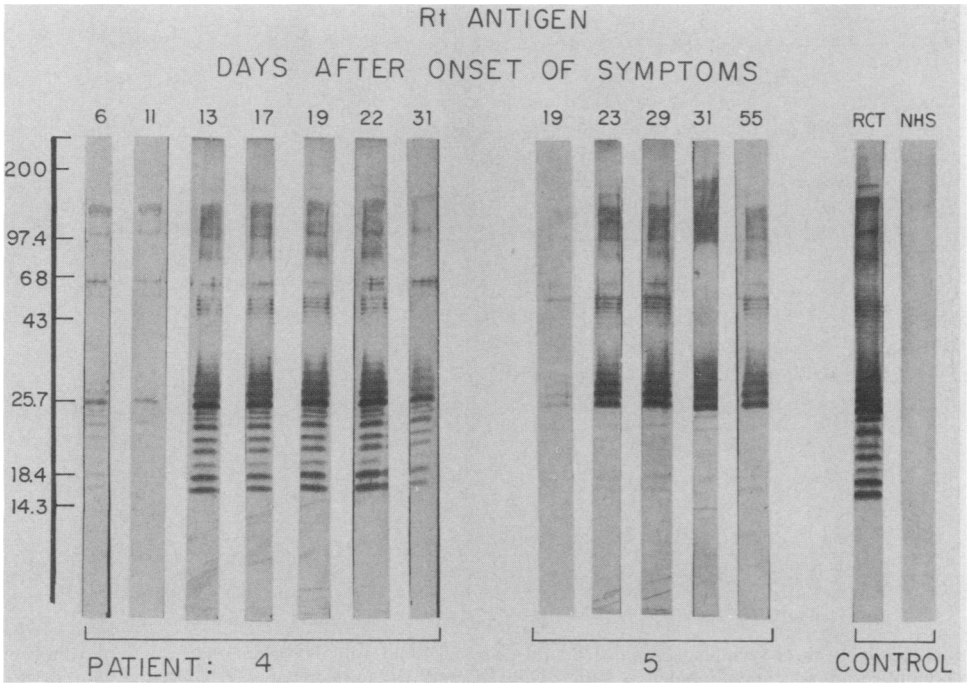

FIG. 2. Immunoblot profile of reactivity of antibodies to *R. typhi* (Rt) in sera obtained at different days after the onset of symptoms for patients 4 and 5. Controls were RCT and nonreactive human serum (NHS) (pool). Numbers are as defined in the legend to Fig. 1.

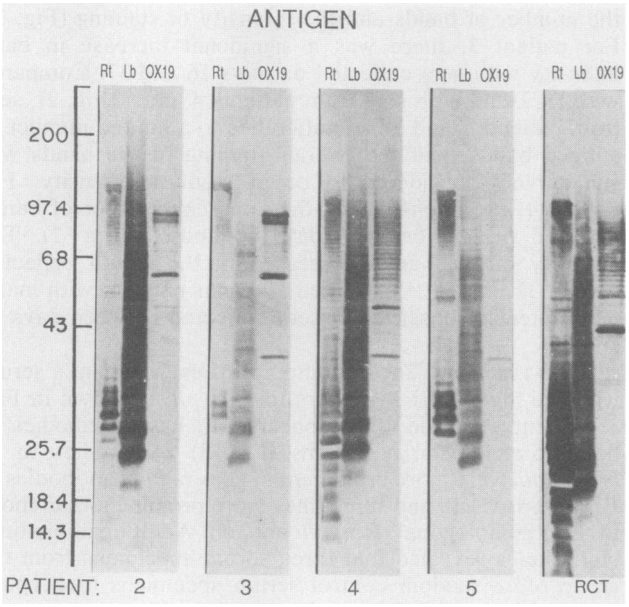

FIG. 3. Immunoblot profile of RCT and one serum specimen from each of the four patients with the antigens of *R. typhi* (Rt), *L. bozemanii* (Lb), and *P. vulgaris* OX19 (OX19). The sera were collected after disease onset: patient 2, 16 days; patient 3, 10 days; patient 4, 17 days; and patient 5, 23 days. The antibodies were probed with HRP–anti-human Ig. Numbers are as defined in the legend to Fig. 1.

from two patients (patients 2 and 4) had IgG antibodies and sera from all patients had IgM antibodies to *L. bozemanii*. Sera from three patients (patients 2, 3, and 4) had IgG antibodies to *P. vulgaris* OX19. Patient 4 also had IgM antibodies and patient 5 apparently did not have either class of antibody to *P. vulgaris* OX19.

Immunoabsorption of patient sera. To determine the specificity of the antibodies which reacted with *R. typhi*, we absorbed the sera with *R. typhi*, *L. bozemanii*, or *P. vulgaris* OX19. When RCT was absorbed (Fig. 6) with *R. typhi*, a significant decrease in band intensity was noted with all three antigens. This result indicated that the antibodies had been absorbed. In contrast, when RCT was absorbed with either *L. bozemanii* or *P. vulgaris* OX19, the reduction in antibodies to *R. typhi* was minimal. Absorption with the combination of *L. bozemanii* and *P. vulgaris* OX19 antigens seemed to remove antibodies to the nonrickettsial antigens (Fig. 6, OX19 + Lb panel). Because of the low number of patient serum specimens available, it was necessary to use the nonrickettsial antigen combination in the absorption step.

After absorption with *R. typhi*, sera from four patients did not react with the *R. typhi* antigen (Fig. 7). The four absorbed sera retained some reactivity with the *L. bozemanii* antigen. For sera from three patients (patients 2, 3, and 5), the reaction with *P. vulgaris* OX19 did not appear to be affected by absorption with the *R. typhi* antigen. The reactivity of the unabsorbed sera from these three patients with *P. vulgaris* OX19 was minimal. The serum from patient 4, however, showed a more extensive reaction with *P. vulgaris* OX19, similar to that observed with the rabbit reactive control sera (data not shown). After absorption of this serum with the *R. typhi* antigen, reactivity with *P. vulgaris* OX19 was also significantly reduced.

Patient sera absorbed with the nonrickettsial antigen mixture showed—as observed with RCT—that antibodies to these antigens were apparently removed. However, antibodies to *R. typhi* were minimally absorbed (Fig. 7).

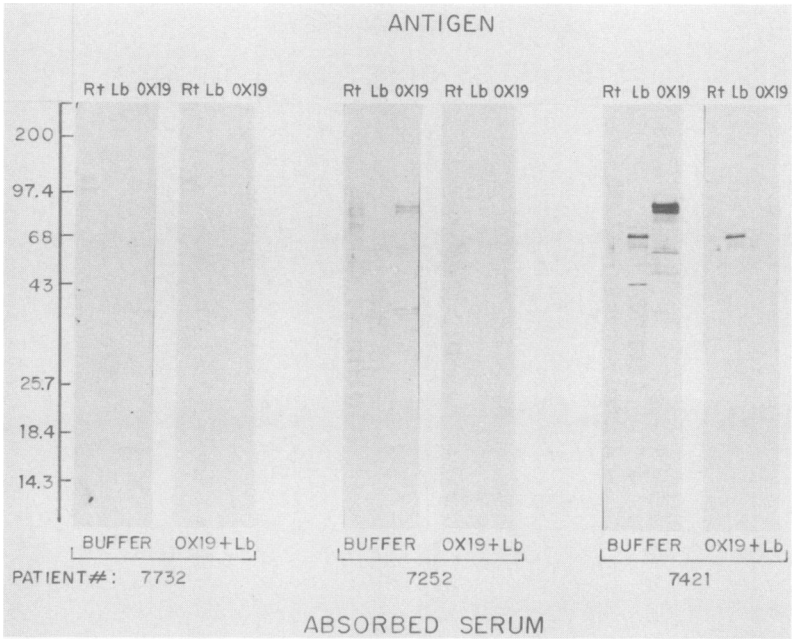

FIG. 4. Immunoblot profile of three serum specimens from the group of 18 nontyphus patient controls. Sera from each of the three patients (first panel for each patient serum specimen, labeled as buffer) were reacted with antigens of *R. typhi* (Rt), *L. bozemanii* (Lb), and *P. vulgaris* OX19 (OX19). The sera were absorbed with a mixture of OX19 and Lb antigens and then incubated with the three antigens (second panel, labeled OX19 + Lb). Numbers are as defined in the legend to Fig. 1.

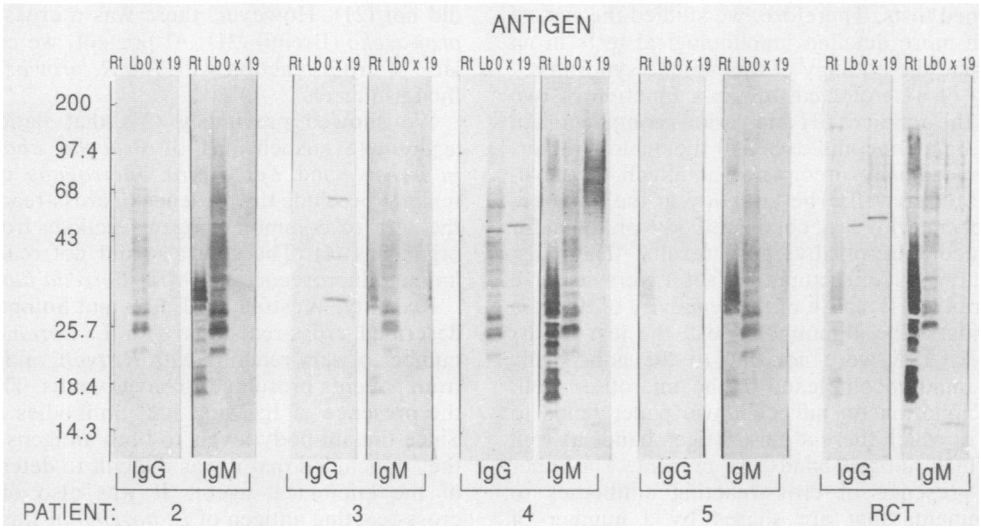

FIG. 5. Immunoblot profile for identifying the specific Ig classes of antibodies. The sera from the four patients and RCT were reacted with antigens of *R. typhi* (Rt), *L. bozemanii* (Lb), and *P. vulgaris* OX19 (0 x 19). Numbers are as defined in the legend to Fig. 1.

DISCUSSION

The diagnosis of infectious diseases is frequently made by serological tests when the causative organism cannot be isolated. In our cases of acute febrile cerebrovasculitis, only low titers of antibodies to rickettsial antigens from the typhus group were detected. The question of the presence of cross-reacting antibodies could not be answered by the

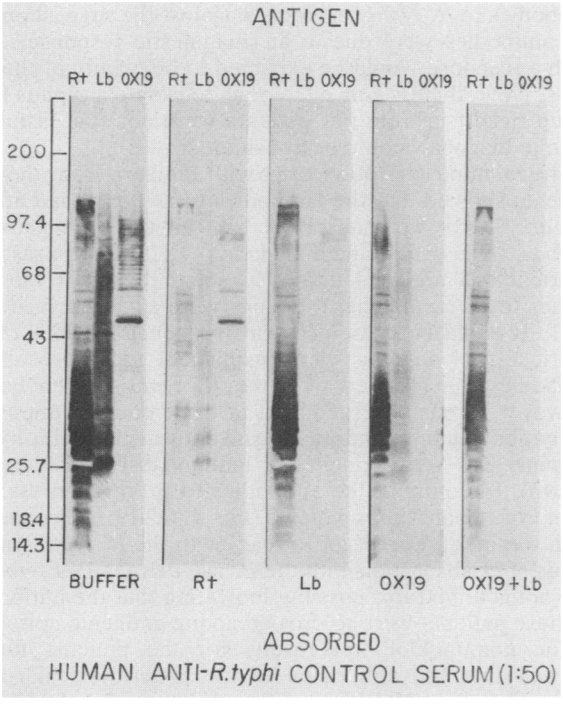

FIG. 6. Immunoblot profile of absorbed RCT. The serum was absorbed with antigens of *R. typhi* (Rt), *L. bozemanii* (Lb), and *P. vulgaris* OX19 (OX19), with a mixture of Lb and OX19 antigens (OX19 + Lb), or with buffer as a control. The absorbed serum was then tested with the three antigens (Rt, Lb, and OX19). Numbers are as defined in the legend to Fig. 1.

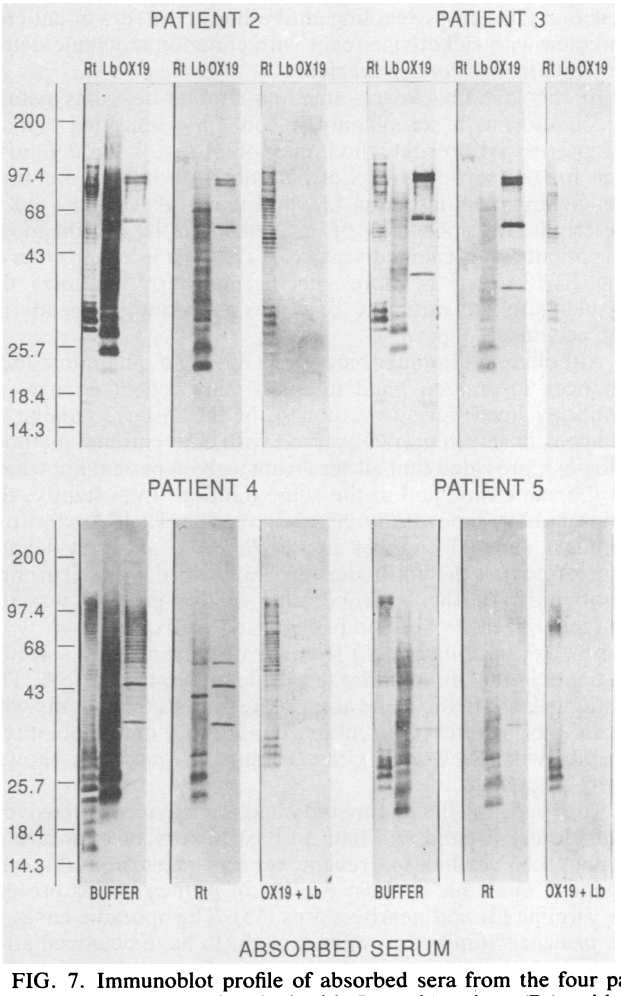

FIG. 7. Immunoblot profile of absorbed sera from the four patients. The sera were absorbed with *R. typhi* antigen (Rt), with a mixture (OX19 + Lb) of antigens of *P. vulgaris* OX19 (OX19) and *L. bozemanii* (Lb), or with buffer as a control. The absorbed serum was then tested with the three antigens (Rt, Lb, and OX19). Numbers are as defined in the legend to Fig. 1.

routinely performed tests. Therefore, we studied the sera of our patients with more detailed immunological tests in an attempt to elucidate the etiology of this unique syndrome.

The specificity of a serological test is a function of two variables. One is the action of pH and various components in patient sera, i.e., immunocomplexes and rheumatoid factor, which may nonspecifically precipitate or attach to the ligands in the microtiter well. The antibody in the immunocomplex then reacts with a second antibody or indicator system to produce false-positive test results. The latex agglutination test results for rheumatoid factor were negative for all patient sera (21). Because of the reactivity of the sera with antigen bands in the immunoblot test, the test results obtained in the ELISA were not due to the nonspecific precipitation of immunocomplexes in the microtiter well. Nonspecific precipitation on nitrocellulose paper tends to occur in regions in which there are no antigen bands as well as in areas in which antigen bands are present. The other variable is the presence of cross-reacting antibodies to antigenic determinants that are shared by a number of unrelated or related pathogens. These antibodies may react specifically with the test substance, but a laboratorian may view them as nonspecific. For example, the Weil-Felix test is one of the few diagnostic tests based on a nonspecific reaction (20); cross-reacting antibodies in the sera of patients infected with rickettsiae react with common antigenic determinants from *Proteus* species.

In the last few years, the immunoblot test has gained recognition as a serodiagnostic tool for confirming clinical diagnoses. At present, the immunoblot test is the definitive test for the serodiagnosis of patients with human immunodeficiency virus infection (2), and it has also been used to determine the specificity of test results in the serodiagnosis of patients with Lyme disease (7). This test is as sensitive as the ELISA but is more specific, because it allows the visualization of antibody reactions with antigen bands on nitrocellulose paper.

Although the immunoblot test is mostly a qualitative tool, changes in antigen band intensity may reflect changes in antibody level during the course of the disease (similar to changes in serum titers observed with conventional methodologies), provided that all sera from a given patient are tested at the same time and at the same dilution. Importantly, the immunoblot time study as well as the ELISAs for our patients showed changes in the *R. typhi* antibody levels, indicating that the antibodies probably were not background antibodies. Further corroboration of this premise was the finding that there were no background antibodies to *R. typhi* (or *L. bozemanii*) in sera from 15 of 18 randomly selected patients in the immunoblot test and none in the ELISA. The patients lived in the same area as the four study patients with acute febrile cerebrovasculitis. In addition, immunoblot test results with *Rickettsia rickettsii* antigen (data not shown) were negative.

The four patients in our study had not traveled extensively outside of Virginia and had no past history of exposure to typhus. Nevertheless, recent reports suggested that the agent of epidemic typhus, *Rickettsia prowazekii*, is present in Virginia (3) and nearby states (15). The sporadic cases of *R. prowazekii* infection were thought to have occurred after contact with flying squirrels (1, 3, 15), from which this agent has been isolated (15). Unlike our patients, however, the 16 patients in two reported series all recovered quickly with therapy, and most were considered to have had mild disease (15). Furthermore, they showed significant serum antibody titers to squirrel isolates of *R. prowazekii*, and our patients did not (21). However, there was a cross-reaction with *R. prowazekii* (Breinl) (21). At present, we cannot explain the lack of cross-reaction with the *R. prowazekii* isolates from flying squirrels.

We showed previously (21) that patient sera did not agglutinate suspensions of *Brucella abortus*, *Francisella tularensis*, and *Leptospira interrogans* organisms. These findings preclude the presence of cross-reacting antibodies in the sera to common antigen fractions from gram-negative organisms (6). The sera also did not react in the indirect immunofluorescence test with *Borrelia burgdorferi*.

Recently, Westfall et al. (22) and Sompolinsky et al. (17) described cross-reactions with *L. bozemanii* antigen in a number of sera reacting with *R. typhi* antigen and obtained from patients presumed to have typhus. They demonstrated the presence of IgG and IgM antibodies to both antigens. Since the antibody levels to both antigens were equivalent, they concluded that it was difficult to determine the identity of the etiological agent. It was also observed that the cross-reacting antigen of *L. bozemanii* was heat and trypsin resistant (17).

The 57 control serum specimens from patients serodiagnosed as having typhus showed extensive cross-reactions with *L. bozemanii*, as did our patient sera. These results may indicate an infection with the rickettsial agent of the typhus group. Moreover, the clinical symptoms described (21) were not compatible with symptoms due to a legionella infection; thus, the disease is unlikely to have been produced by *L. bozemanii*. Immunoblot studies with *Legionella pneumophila* and *Legionella micdadei* antigens were negative with the sera from the four patients (data not shown). The presence of antibody to *R. typhi* mainly of the IgM class may also indicate that the infection was recent and could have been due to *R. typhi*. Moreover, the presence of IgM antibodies to *R. typhi* also argues against the suggestion that the antibodies were due to an anamnestic response, since such a response would be expected to be mainly of the IgG class. Nevertheless, since attempts to isolate a typhus fever group rickettsia from our patients were not successful, no definite diagnosis could be considered.

The immunoabsorption tests with the sera from the four patients showed that the antibodies to *L. bozemanii* and *P. vulgaris* OX19 were absorbed with all three antigens, *R. typhi*, *L. bozemanii*, and *P. vulgaris* OX19. In contrast, the nonrickettsial antigens did not substantially absorb the antibodies to *R. typhi*. These findings were also observed with RCT. It could be presumed that the concentrations of the nonrickettsial antigens were insufficient to absorb all the antibodies that reacted with the *R. typhi* antigen bands. However, it was observed that, at these concentrations, all the antibodies to the nonrickettsial antigens in rabbit hyperimmune sera were completely immunoabsorbed (data not shown). In contrast, the antibodies to *R. typhi* in the sera from 3 of 18 nontyphus patients (Fig. 4, OX19 + Lb panel for each serum) appeared not to react with the *R. typhi* antigen bands after absorption with the *P. vulgaris* OX19-*L. bozemanii* antigen mixture, possibly indicating that the antibodies in these patients were to cross-reacting antigenic epitopes.

The immunoblot test results for the patients further showed that the antibodies to the lower-molecular-mass antigen bands (<25.5 kDa) of *R. typhi* were absorbed by the nonrickettsial antigens, indicating that most of the epitopes were cross-reacting epitopes. In contrast, the sera absorbed with the nonrickettsial antigens reacted with the higher-molecular-mass antigen bands of *R. typhi*, indicating that these antigen bands probably contained cross-reacting

epitopes as well as epitopes specific for the *R. typhi* antigen. Therefore, it was difficult to assign to the typhus antigen a specific antigen band which could conclusively indicate that these patients had a typhus infection.

Thus, the results of the absorption studies are consistent with our premise that the patients may have had an infection with a typhus fever group rickettsia. It is of interest that Linnemann et al. (13) recently reported the case of a 37-year-old woman from southwestern Ohio with a syndrome compatible with acute febrile cerebrovasculitis. Detailed serological analyses with enzyme immunoassays and protein immunoblots were strongly suggestive of an infection with a non-spotted fever group rickettsia. It is likely, therefore, that the syndrome we described is not confined to the Piedmont Region of Virginia.

We conclude that neither background antibodies to rickettsiae nor cross-reacting antibodies to common gram-negative bacteria or to *L. bozemanii* can explain the serological responses in our patients with acute febrile cerebrovasculitis. A typhuslike agent or an unrelated unknown agent which shares common epitopes with *R. typhi* may be the cause of the syndrome.

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