Sensitive Enzyme-Linked Immunosorbent Assay for Detection of Antibodies Against Typhus Rickettsiae, *Rickettsia prowazekii* and *Rickettsia typhi*

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An enzyme-linked immunosorbent assay (ELISA) has been developed for the titration of rickettsial antibodies in human and animal sera. Two preparations of soluble typhus-group antigens were obtained from *Rickettsia typhi* and *Rickettsia prowazekii* by ether extraction: a standard antigen from infected yolk sacs (YS antigen) and one free of yolk sac contaminants from Renografin-purified rickettsiae (PR antigen). Rabbit, mouse, and guinea pig sera were obtained by immunization with viable purified *R. typhi* or *R. prowazekii*. Human sera were obtained from individuals who had recovered from laboratory infections with either typhus rickettsia months or years previously. Goat-derived anti-immunoglobulins were conjugated to alkaline phosphatase with glutaraldehyde. Although the PR and YS antigens gave equivalent antibody titers in the complement fixation test, the PR antigen was clearly superior in the ELISA. With this antigen, the titration curves of all antisera were linear over a wider range of serum concentrations and the titers were higher than with the YS antigen. With YS and PR antigens, ELISA titers were higher than those obtained by complement fixation by one and two orders of magnitude, respectively. In human sera, immunoglobulin G and immunoglobulin M antibodies were demonstrated by their respective anti-immunoglobulins and by differential susceptibility to ethanethiol. ELISA titers showed some type specificity, whereas none was observed in complement fixation tests. The ELISA is highly sensitive, reproducible, and easily adaptable to the various requirements of clinical and research laboratories.

As Ormsbee (20) has pointed out, several techniques are available for the detection and assay of antibodies against rickettsiae, but each has certain limitations. Of the tests with rickettsial antigens, the complement fixation (CF) is the most widely used. It can be performed with soluble antigens or with repeatedly washed rickettsiae. The former antigens are group specific in the case of the typhus and spotted fever groups, whereas the latter display considerable species specificity or, in the case of scrub typhus rickettsiae, type specificity. The key to a satisfactory CF test is careful preparation of antigens, but these cannot always be easily standardized for maximum reactivity and specificity (2, 28). Particulate antigens employed in the CF can also be used in the microagglutination (MA) test (8), which is much simpler to perform and more sensitive than the CF test. Of considerable value in rickettsial serology is the indirect immunofluorescence procedure, especially since the development of the microimmunofluorescence technique (21-23).

This test is also carried out with particulate antigens. Although more cumbersome than the MA test, it is highly sensitive, has the added advantage that it can directly identify antibodies as immunoglobulin G (IgG) or IgM, and is valuable for serotyping procedures (34). The indirect hemagglutination test is relatively simple and sensitive, but has the disadvantage that the erythrocyte-sensitizing substance is a rather specialized antigen that survives boiling in strong alkali (3). It was recently shown to be of considerable value with human sera, but variable results were obtained with immune rodent sera (1, 22, 29).

Despite the above-described advances in rickettsial serology, there is still a need for a versatile procedure that possesses all of the advantages of the other tests, but can also be used to provide quantitative measurements of antigen or antibody content or to serve as a tool for detailed antigenic or antibody analysis.

Since the introduction and adaptation to the measurement of antibody of the enzyme-linked

immunosorbent assay (ELISA) by Engvall and Perlmann (6, 7), its broad applicability and potential for immunological detection have been widely recognized (33, 36). The method has proven to be a convenient, reliable, and highly sensitive serological tool in diverse clinical and research areas. These useful attributes, as well as our recent demonstration that highly satisfactory antigen can be obtained from typhus rickettsiae purified by Renografin density gradient centrifugation (4), encouraged our efforts to introduce the ELISA procedure to rickettsial serology. We describe here its successful use for the detection of antibodies against soluble typhus-group rickettsial antigens in sera from human and several animal species.

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MATERIALS AND METHODS

Preparation of antigens. Soluble Renografin-purified (PR) antigens were obtained from Renografin density gradient-purified *Rickettsia typhi* (Wilmington) and *Rickettsia prowazekii* (E and Breinl strains) by ether extraction as described previously (4). The Formalin-treated, purified rickettsiae (not treated with ether) were used as antigens in MA tests. Standard yolk sac (YS) soluble antigens were prepared directly from infected frozen yolk sacs. The yolk sacs were first macerated by shaking with glass beads in brain heart infusion broth and then centrifuged at 8,000 rpm for 30 min in a Sorvall GSA rotor. The fat and supernatants were discarded, and the pellet was suspended in 0.145 M NaCl, 0.04 M potassium phosphate, 0.5% Formalin (pH 7.2) buffer (FBS) for 24 to 72 h to inactivate the rickettsiae. The suspension was then centrifuged at 8,000 rpm for 30 min, and the supernatant and fat were again discarded. After suspending the pellet in 10 ml of FBS per g (wet weight) of pellet, the suspension was extracted twice with equal volumes of ether. The aqueous phase was then centrifuged at 8,000 rpm for 30 min to pellet the particulate rickettsial antigen. The pelleted particulate antigen was washed twice by centrifugation in 1 M KCl-0.04 M potassium phosphate (pH 7.0) before suspension in FBS for use in the MA tests. The final standard soluble antigen was obtained by bubbling filtered air through the supernatant to remove dissolved ether and removing residual particulates by a final centrifugation at 8,000 rpm for 30 min. Control yolk sac antigens were prepared similarly from uninfected yolk sacs. Protein concentrations were determined by the method of Lowry et al. (13).

Preparation of animal antisera. The preparation of hyperimmune rabbit antisera against both uninfected yolk sacs and Renografin-purified *R. typhi* (Wilmington) and *R. prowazekii* (E strain) has been described (4, 35). Similarly, male 350-g Naval Medical Research Institute (Nmri: [HA] CV) guinea pigs were immunized with a single intraperitoneal inoc-

ulation of purified viable rickettsiae (10 μ g of protein) in 0.5 ml of Dulbecco phosphate-buffered saline and bled by heart puncture 21 days later. Pools of immune and hyperimmune mouse sera were also obtained by inoculating purified viable rickettsiae intraperitoneally into 3-week-old NMRI mice (NIH/NMRI CV). Only toxic deaths occurred in mice immunized with the E strain of *R. prowazekii* (14 to 22 μ g of protein per mouse in 0.1 ml of Dulbecco phosphate-buffered saline, about 0.8 mouse 50% lethal dose). They were bled at 21 days by heart puncture, hyperimmunized at 28 days with 10 μ g of frozen rickettsial protein per mouse and bled again at 35 or 50 days after the initial injection. To prevent deaths due to infection, mice immunized with *R. typhi* (0.15 μ g of rickettsial protein per mouse) required an initial maintenance on oral tetracycline hydrochloride (approximately 0.8 g/50 ml of water) (Polyotic; American Cyanamide Co., Princeton, N.J.) for 2 weeks. They were then bled at 21 days, hyperimmunized with 1.5 μ g of frozen rickettsial protein per mouse, and bled again at 35 days. The purified viable rickettsiae used for immunization did not contain demonstrable yolk sac antigens. The rabbit immune sera did not react with yolk sac antigens (4, 35).

Source of human antisera. Sera from several late-convalescent cases of laboratory infections with typhus rickettsiae were provided to us through the courtesy of Charles L. Wisseman, Jr., Department of Microbiology, University of Maryland Medical School, Baltimore.

Serological tests. MA tests employed both Formalin-treated, Renografin-purified rickettsiae and the crude particulate antigen obtained during the other method of preparation of soluble antigen described above. Antigens were standardized to an optical density (OD) of 2.00 at 420 nm and diluted with 0.5 volume of 1% normal rabbit serum in 0.85% NaCl. One drop (25 μ l) of antigen was mixed with 1 drop of serum dilution in saline containing 0.5% normal rabbit serum, and the mixture was incubated overnight at room temperature as described by Fiset et al. (8). The test was read without acridine orange staining. Standard microtiter methods were used in CF tests (27, 32). Only Formalin-treated soluble antigens were used, and all antisera were inactivated at 60°C for 30 min. Weil-Felix slide tests were performed with Difco antigens on all sera. Tube tests were used to quantitate all sera showing any slide agglutination. All titers were recorded as the final serum dilution before antigen addition.

Enzyme-linked antiglobulin conjugates. The IgG fraction of goat antiserum against immunoglobulin fractions of each species was conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) by the method of Engvall and Perlmann (7). Briefly, the method consisted of conjugating the immunoglobulin fractions to the enzyme, in ratios of 1:3 (wt/wt), by addition of glutaraldehyde (0.2% final) and incubation at room temperature for 2 h. One milliliter of conjugate contained in 0.05 M sodium phosphate-buffered saline (pH 7.4) containing 0.001 M $MgCl_2$ was dialyzed overnight against 1 liter of phosphate-buffered saline and then for 8 h against

0.036 M sodium borate-buffered saline (pH 7.85) (BBS) containing 0.01% sodium azide. Undiluted conjugates were stored at 4°C for as long as 6 months without noticeable loss in titer. The following IgG fractions of individual goat antispecies immunoglobulins were purchased from Microbiological Associates, Inc., Bethesda, Md.: rabbit immunoglobulins (catalog no. 63-386); guinea pig gamma globulin (catalog no. 63-324); mouse IgG (catalog no. 63-368); human IgM (catalog no. 61-147); and human IgG (catalog no. 61-130).

Conjugates were diluted immediately before use. Dilutions were determined for each conjugate by titration against positive and negative (control) reference sera. The lowest conjugate concentration that elicited a high absorbance value (0.8 to 1.0 OD) with the positive serum and a low value (<0.1 OD) with the negative serum was used for all experiments.

ELISA. The method originally described by Engvall and Perlmann (7) and adapted by Schinski et al. (26) was used with minor modifications. The standard procedure was as follows. Soluble antigen preparations diluted in coating buffer (0.1 M sodium carbonate-0.02% NaN_3 , pH 9.6) were dispensed in 1-ml amounts into disposable polystyrene tubes (10 by 75 mm; Falcon 2038) and incubated overnight at 37°C in a shaking water bath. The fluid was aspirated, and the sensitized tubes were washed two times with 2 ml of BBS containing 0.5% bovine serum albumin fraction V (Calbiochem, La Jolla, Calif.), 0.5% Tween 20 (Sigma), and 0.01% NaN_3 (BBS-AT). One milliliter of antiserum diluted in BBS-AT was then added, and the tubes were incubated for 2 h at 37°C. After two washes with BBS-AT, 1 ml of enzyme conjugate diluted in BBS-AT was added and incubated for 2 h at 37°C. Finally, reaction tubes were washed twice with BBS-AT and once with substrate buffer (0.05 M sodium carbonate and 0.001 M MgCl_2 , pH 9.8) before assaying for alkaline-phosphatase activity by adding 1 ml of p-nitrophenyl phosphate (Sigma; 1 mg/ml) dissolved in substrate buffer. Tubes were then returned to the shaking water bath (37°C) for 30 min, and the reaction was stopped by the addition of 1 ml of 1 N NaOH. Absorbance was read at 400 nm in a spectrophotometer (DB-GT; Beckman Instruments, Inc., Fullerton, Calif.).

All assays were done in duplicate, and the means were determined. The duplicates generally agreed within 0.02 absorbance units. Less than 15% differences in absorbance were observed from day to day with the positive and negative control sera included in each experimental series. Complete ELISA titration curves were obtained for each serum. The ELISA end-point titer chosen was that dilution of serum (obtained from the titration curve by interpolation) that gave a 0.30 OD value, about seven times those of the usual negative serum controls.

Ethanol treatment. Undiluted human convalescent sera were treated with 0.03 M ethanolol (Eastman Organic Chemical Div., Eastman Kodak Co., Rochester, N.Y.) for 3 h according to the procedure described by Murray et al. (18, 19) and used immediately in the MA test. Samples were allowed

to stand uncapped for 30 min at 37°C to allow residual reagent to evaporate before use in the ELISA.

RESULTS

Characterization of the soluble antigen fractions of typhus rickettsiae. Rickettsial antigens are usually prepared from crude yolk sac suspensions (31), but Dasch and Weiss (4) have shown that highly satisfactory antigens can also be obtained from Renografin-purified rickettsiae. For these studies, soluble antigens were derived from both sources by the standard diethyl ether extraction procedure. Table 1 presents for each antigen the titer as determined by CF and the concentration required for optimal binding to the polystyrene tubes for the ELISA. Each preparation contained several antigenic components (24), each of unknown chemical composition and differing in the amount of material not antigenic with the rickettsial antisera. Consequently, although the protein content of each antigen is given, the results are expressed in terms of antigen dilution.

Two rabbit antisera, no. 7 prepared against *R. typhi* and no. 10 prepared against the E strain of *R. prowazekii*, were employed to titrate the antigens of each strain: Wilmington, E, and Breinl. Grid titrations of PR and YS antigens of each species gave virtually identical patterns with each serum. The results obtained with 8 U of serum provided for optimal sensitivity of antigen detection by CF. The YS and PR antigens had similar CF titers, suggesting similarity in total content of rickettsial antigen, despite pronounced differences in protein content. This difference was probably due to the relatively high amount of contaminating yolk sac proteins in the YS antigens (9, 10, 16). The antigen titers were approximately the same with the homologous and heterologous antisera.

Despite their similar CF titers, the PR and YS antigen fractions had very different ELISA antigen titration curves (Fig. 1). In each case, the YS antigen had a shallow titration curve with relatively low OD values even at the optimal dilutions. In contrast, PR antigen titration curves were bell-shaped, falling off at high and low antigen concentrations and having high OD values. The OD values at optimal dilution were 0.4 to 0.7 OD units higher for PR antigens than the corresponding peak values for YS antigens. Although the ELISA titrations of soluble antigens were usually done at an antibody dilution (1:4,000) that gave useful OD values, antigens titrated with higher antibody concentrations gave similar titration curves and opti-

TABLE 1. Characteristics of typhus-group soluble antigens

Antigen		CF test ^a		ELISA			
Source	Protein (μg/ml)	<i>R. typhi</i> serum no. 7	<i>R. prowazekii</i> se- rum no. 10	<i>R. typhi</i> serum no. 7		<i>R. prowazekii</i> serum no. 10	
				Optimal dilution ^b	OD ^c	Optimal dilution	OD
<i>R. prowazekii</i>							
Renografin purified							
E	23	40	20	50	0.84	50	0.86
Breinl	45	64	64	100	0.94	250	0.96
Standard yolk sac							
E	105	40	40	500	0.32	500	0.39
Breinl	135	64	32	500	0.58	500	0.55
<i>R. typhi</i> (Wilmington)							
Renografin purified							
No. 1	15	40	40	100	0.93	100	0.78
No. 2	25	80	160	100	1.22	250	1.19
No. 3	35	64	64	250	1.21	250	1.24
Standard yolk sac	165	80	80	500	0.54	500	0.42

^a Reciprocal of greatest antigen dilution giving 30% hemolysis with 5 U (50% end point) of complement and 8 U of each rabbit antiserum. The titers of the antisera are shown in Table 2.

^b Reciprocal of antigen dilution giving greatest OD value with rabbit antisera diluted 1:4,000.

^c OD (400 nm) at optimum dilution.

FIG. 1. Determination of optimal antigen dilutions by ELISA. Hyperimmune rabbit sera against *R. typhi* (no. 7) (○) and *R. prowazekii* strain E (no. 10) (●) were each diluted to 1:4,000, and anti-yolk sac serum (▲) was diluted to 1:400. Goat anti-rabbit immunoglobulin alkaline phosphatase conjugate was diluted to 1:4,000. CF titers and protein contents of antigens are presented in Table 1. *R. typhi* PR antigen no. 1 was used (see Table 1).

mal dilutions of antigen. The same was true of antigen titrations with guinea pig and human sera.

The reduction in YS-antigen binding at high antigen concentrations was probably due to nonantigenic material of yolk sac origin competing for space on the tube surface. Rabbit

antiserum was prepared against uninfected yolk sacs and was used at a 1:400 dilution to measure directly the binding of yolk sac antigens present in the PR (Fig. 1C) and YS (Fig. 1D) antigens of *R. prowazekii* strain E. Bound yolk sac antigens were not detected in the PR antigen. The YS antigen, however, had signifi-

cant amounts of yolk sac material that bound to the tubes. Similar findings (not shown) were made with the antigen of *R. typhi*. The titration curve (not shown) of a soluble antigen prepared by ether extraction of uninfected yolk sacs, when titrated with anti-yolk sac serum, was very similar to those of standard YS antigens. A reconstruction experiment, in which this soluble yolk sac antigen was added to PR antigen and titrated against antirickettsial serum, also demonstrated a very significant inhibition of binding of rickettsial antigen by yolk sac material. At the concentrations used here, uninfected yolk sac material did not inhibit the phosphatase reaction.

Optimal ELISA concentrations of each antigen were generally identical whether rabbit antiserum against *R. typhi* or *R. prowazekii* was used (Table 1). When they differed, the higher concentration of antigen was used in all antiserum titrations described below.

Titration of immune and hyperimmune animal sera. Differences between the PR and YS soluble antigens of *R. typhi* or *R. prowazekii*, demonstrated in antigen titrations by the ELISA (Fig. 1), were also seen in all serum titrations. Figures 2A and B illustrate titration curves for high- and low-titer homologous and heterologous rabbit hyperimmune sera against soluble *R. typhi* PR and YS antigens, respectively. With the PR antigen, the slopes were generally linear over a range of 10^{-5} to 10^{-3} antiserum dilutions, and at concentrations above 10^{-3} the OD readings were often significantly above 1.0 OD. In contrast, antiserum titrations against YS antigen did not attain the OD values seen with the PR antigen, even at antibody dilutions of 1:100. Often only a broad peak of low OD was reached at low dilutions (10^{-2} to 10^{-3}).

Titration curves of guinea pig immune and mouse hyperimmune sera similar to those shown in Fig. 2 were obtained with PR antigens, but results with YS antigens were less satisfactory in these species. Shallow titration curves above control values were obtained with YS antigens, but some curves did not reach the 0.30 OD end point, even after extending the enzyme reaction time to 60 min. Differences between PR and YS antigens were not demonstrated by CF or MA tests with any of the sera.

Table 2 presents the titers of the rabbit sera determined by CF and ELISA with six antigens: PR and YS from *R. typhi* and from *R. prowazekii* strains E and Breinl. CF titers of the sera were similar whether 2, 4, or 8 U of each antigen was employed in the test. ELISA titers against YS antigens were 2.5 to 33 (mean, 14) times the corresponding CF titers.

FIG. 2. Titration by ELISA of rabbit antisera using *R. typhi* soluble antigens. (A) Renografin-purified antigen no. 1 (1:100 dilution). (B) Standard yolk sac antigen (1:500 dilution). Sera against *R. typhi*: no. 6 (○); no. 7 (△). Sera against *R. prowazekii* strain E: no. 9 (◆); no. 11 (■). Sera against *R. tsutsugamushi* strain Karp: (×). Goat anti-rabbit immunoglobulin enzyme conjugate was used at a 1:4,000 dilution.

When PR antigens were used, an even greater sensitivity was demonstrated: the average ELISA titer was approximately 45 times the CF titer (range, 18 to 120). No rickettsial species specificity was demonstrated in CF tests (Table 2), whereas only the anti-*R. typhi* sera gave distinctly higher titers against homologous antigens in MA tests (data not shown). By the ELISA, rabbit antisera against *R. typhi* had as much as fourfold variations in titer against different antigen preparations without consistent species specificity. Anti-*R. prowazekii* sera tested with PR antigens, on the other hand, had consistently higher titers (usually two- to threefold) with homologous antigens. When hyperimmune rabbit antiserum prepared against the Karp strain of *Rickettsia tsutsugamushi* was titrated against *R. typhi* YS antigen, only the very low negative control serum values were obtained by ELISA (Fig. 2B). Negative control values were also obtained against *R. typhi* PR antigen with a Difco reference antiserum prepared against *Proteus* OX19 antigen for

use in the Weil-Felix test.

Table 3 presents MA, CF, and ELISA titers of immune guinea pig antisera against the PR and YS antigens of *R. typhi* and *R. prowazekii* (Breinl). ELISA titers with PR antigens were higher than those obtained by CF and MA tests by approximately the same magnitude as with rabbit sera. ELISA titers with YS antigen, however, were variable and only slightly greater than the MA and CF titers. Although the guinea pig sera were similar to the rabbit sera in that only the anti-*R. typhi* sera showed any species specificity in the MA test, both sets of guinea pig sera, anti-*R. typhi* and anti-*R. prowazekii*, reacted specifically in the ELISA,

displaying 1.4- to 10-fold-higher titers against their homologous PR antigens.

MA, CF, and ELISA titrations of serial pools of antiserum against *R. typhi* or *R. prowazekii* from immune and hyperimmune mice were also done (data not shown). MA titers were generally 8 to 16 times higher than CF titers. Although the hyperimmune mouse sera gave excellent ELISA IgG titers against PR antigens (25 to 100 times the CF titers), the immune sera (up to 21 days after a single inoculation) reacted weakly (less than 0.30 OD) even at 1:100 dilutions, despite CF titers of 8 to 32 and MA titers of 16 to 512. Whether antityphus immune mouse sera contained predominately IgM and

TABLE 2. *Titers of rabbit typhus-group antisera*^a

Antisera	CF ^b						ELISA					
	PR antigen			YS antigen			PR antigen			YS antigen		
	<i>R. typhi</i> no. 1		<i>R. prowazekii</i>	<i>R. typhi</i>		<i>R. prowazekii</i>	<i>R. typhi</i> no. 1		<i>R. prowazekii</i>	<i>R. typhi</i>		<i>R. prowazekii</i>
	E	Breinl		E	Breinl		E	Breinl		E	Breinl	
<i>R. typhi</i>												
No. 5	1,280	640	1,280	640	640	1,280	23,000	19,000	24,000	6,600	1,600	6,600
No. 6	640	320	640	640	320	640	14,500	10,000	18,000	2,700	2,500	2,600
No. 7	640	640	640	640	640	640	27,800	24,500	37,500	15,200	8,400	21,000
No. 8	640	320	640	640	640	640	16,800	14,000	18,500	9,000	11,500	14,000
<i>R. prowazekii</i>												
No. 9	80	320	160	80	160	160	3,600	14,500	8,000	2,200	1,300	2,800
No. 10	640	640	640	640	640	640	22,000	31,000	53,000	6,100	12,000	4,200
No. 11	640	640	640	640	640	640	25,500	69,000	76,000	20,000	13,300	20,000
No. 12	320	640	320	320	320	320	8,200	46,000	19,500	800	2,500	1,500

^a Titers were expressed as reciprocals of serum dilutions. ELISA titers were interpolated at 0.30 OD. The E strain was used to elicit the *R. prowazekii* antisera.

^b Highest titer obtained with 2, 4, and 8 U of each antigen (Table 1).

TABLE 3. *Titers of guinea pig typhus-group antisera*^a

Antiserum	MA				CF				ELISA			
	PR antigen		YS antigen		PR antigen		YS antigen		PR antigen		YS antigen ^b	
	<i>R. typhi</i>		<i>R. prowazekii</i>		<i>R. typhi</i>		<i>R. prowazekii</i>		<i>R. typhi</i>		<i>R. prowazekii</i>	
	<i>R. typhi</i>	<i>R. prowazekii</i> ^c	<i>R. typhi</i>	<i>R. prowazekii</i>	<i>R. typhi</i> ^d	<i>R. prowazekii</i>	<i>R. typhi</i>	<i>R. prowazekii</i>	<i>R. typhi</i> ^d	<i>R. prowazekii</i>	<i>R. typhi</i>	<i>R. prowazekii</i>
<i>R. typhi</i>												
No. 5	1,024	64	512	128	128	128	128	128	5,000	950	560	160
No. 6	1,024	128	1,024	128	512	128	256	128	13,000	1,400	ND ^e	ND
No. 7	1,024	128	1,024	128	256	128	256	256	7,800	2,800	ND	ND
No. 8	1,024	128	1,024	256	1,024	512	1,024	1,024	48,000	8,000	3,900	<100
<i>R. prowazekii</i>												
No. 1	32	32	64	64	64	32	32	64	860	3,600	ND	ND
No. 2	32	32	32	64	64	64	64	128	2,700	3,700	550	700
No. 3	64	64	32	64	128	128	64	64	1,300	3,700	ND	ND
No. 4	64	64	64	64	128	128	128	128	340	3,400	<100	<100

^a Titers were expressed as reciprocals of serum dilutions. ELISA titers were interpolated at 0.30 OD. CF titers are the highest obtained with 2, 4, and 8 U of each antigen (Table 1).

^b Enzyme reaction time extended to 60 min.

^c Breinl strain antigen in all titrations.

^d Antigen no. 2. See Table 1.

^e ND, Not done.

only low levels of IgG is under investigation. Rickettsial species specificity of the mouse antisera could easily be demonstrated by MA and ELISA procedures, whereas only questionably significant twofold differences were apparent in the CF test.

Titration of typhus-group human sera. Seven sera from five cases of human laboratory infections with typhus rickettsiae were titrated by the CF and MA tests and by ELISA, using goat anti-human IgM and IgG conjugates (Table 4). Titers obtained against PR or YS antigens by MA or CF tests were again very similar. MA tests generally yielded titers that were two to eight times those obtained by the CF test. Both IgM and IgG ELISA titers could be readily obtained with PR or YS antigens, but titers with PR antigens were distinctly higher, and the OD in ELISA IgG titrations varied directly with the concentration of serum over a wider range. When IgM was titrated, lower ELISA titers were obtained in most cases with PR and YS antigens, as would be expected from late-convalescent sera. IgM and IgG ELISA titers with PR antigens were 2 to 130 and 70 to 300 times the CF titers, respectively. Since the sera were inactivated at 60°C for the CF test, it can be assumed that the CF test measured primarily IgG antibodies (18).

Since all of the individuals had been exposed repeatedly to both *R. typhi* and *R. prowazekii*, little can be said with certainty about the species specificity of the reactions obtained with these sera. However, serum no. 1 was from a repeatedly immunized individual exposed to infection for over 25 years and had the highest IgG and lowest IgM titers. Serum no. 5 was taken the shortest time from onset of an *R. prowazekii* infection and had the highest IgM and lowest IgG titer. This was also the only serum with a positive Weil-Felix test (1:160). Serum no. 4 was from a person who had been immunized with *R. prowazekii* 6 years before infection with *R. typhi* and had uniformly higher titers against *R. prowazekii* by the ELISA. In contrast, two other paired sera (no. 2 and 3) from *R. typhi* infections had uniformly higher *R. typhi* than *R. prowazekii* titers by ELISA and declining titers of both IgM and IgG. Since MA and ELISA tests were done with different antigens, they were not expected to yield identical results.

Ethanethiol treatment. Ethanethiol treatment has been used to selectively destroy IgM in order to demonstrate the presence of this immunoglobulin class in immune sera (18). It was of interest to determine if this treatment was compatible with the ELISA procedure. All seven samples of human convalescent sera

TABLE 4. Titers of human typhus-group antisera^a

Antiserum no.	MA				CF				ELISA IgM				ELISA IgG			
	PR antigen		YS antigen		PR antigen		YS antigen		PR antigen		YS antigen		PR antigen		YS antigen	
	<i>R. typhi</i> <i>prowazekii</i> ^b	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>	<i>R. typhi</i> <i>prowazekii</i>
1	1,024	128	256	256	64	64	64	64	130	200	<100	<100	17,500	19,500	1,150	1,400
2 ^a	64	32	64	32	64	64	32	32	870	620	180	110	4,400	1,900	1,700	550
2 ^b	32	16	16	16	16	16	16	8	230	300	<100	<100	1,400	700	350	350
3 ^a	64	32	64	32	16	16	8	8	1,100	830	400	520	2,100	880	630	720
3 ^b	128	32	64	32	8	8	8	8	400	400	<100	<100	730	370	370	600
4	128	32	64	32	32	32	32	64	360	470	210	320	6,300	14,500	1,700	1,900
5	64	32	64	128	16	16	8	16	2,100	1,500	950	650	1,000	670	460	100

^a Titers were expressed as reciprocals of serum dilutions. ELISA titers were extrapolated at 0.30 OD unit. CF titers are the highest obtained with 2, 4, and 8 U of each antigen (Table 1).

^b Breinl strain antigen in all titrations.

were treated with ethanethiol and titrated against *R. typhi* PR antigen. Two representative results, one with a high IgG/IgM ratio and another with a lower IgG/IgM ratio, are shown in Fig. 3. The IgG titers were not significantly affected in either case, whereas the IgM titers were essentially eliminated in both instances, as was also found with the other five sera. MA titers also decreased after ethanethiol treatment in sera with significant IgM titers demonstrated by the ELISA.

DISCUSSION

Although application of the ELISA procedure to the detection and measurement of antibodies against bacterial antigens is progressing rapidly (33, 36) and has recently been extended to the trench fever agent, *Rochalimaea quintana* (11), to our knowledge the efforts described here are the first with the genus *Rickettsia*. Since the ELISA requires careful examination of the properties of any new set of antigens, our efforts were confined to the tube test and to antigens solubilized from typhus rickettsiae by diethyl ether. It was shown, however, in more recent experiments (unpublished data) that the test can be carried out in microplates without sacrifice of precision and with other antigenic fractions derived from purified rickettsiae.

The antigen titration curves with PR antigens shown in Fig. 1 are comparable to those obtained with other bacterial antigens (12, 30), despite obvious differences in degree of heterogeneity. However, PR antigens were decidedly superior to YS antigens in the ELISA with all antisera. Although usable with rabbit and human antisera, the YS antigens were unsatisfactory with guinea pig or mouse antisera under

our standard conditions. These differences were not noted in the CF test, possibly because of less interference with contaminating yolk sac material or less sensitivity. Although numerous attempts have been made to purify particulate rickettsial antigens (35), efforts to remove yolk sac material from soluble YS antigens have been relatively few. Ammonium sulfate fractionation and diethylaminoethyl chromatography, applied by some investigators to YS antigens (9, 14, 15), deserve further examination. These methods may improve the usefulness of these antigens in the ELISA and present an alternative to Renografin purification (35). On the other hand, Renografin purification may offer some important advantages, since it was shown in a previous study (4), as well as in this one, that significant amounts of soluble antigens are retained. Ether extraction, which is essential with rickettsial suspensions that are heavily contaminated with yolk sac, is a very wasteful method for extracting antigens from purified rickettsiae. Consequently, improved extraction methods may offset the cost incurred in purifying the rickettsiae. In any case, the near identity of the titers obtained in the CF test with YS and PR antigens and the similarity in the ranking of sera by ELISA with these two antigens suggest that the compositions of YS and PR antigens of rickettsial origin are very similar. Either one could be used clinically.

Our ELISA procedure was significantly more sensitive than the CF and MA tests for all sera tested. The quantity of PR antigen required for testing one serum dilution by the ELISA tube method is about 0.5 μ g (in contrast to 6 μ g for the MA test and 0.01 to 0.10 μ g for the CF test). This amount can be further reduced in the mi-

FIG. 3. Effect of ethanethiol treatment on IgM and IgG antibodies in convalescent human sera determined by ELISA titration. Untreated sera (—); treated sera (---). Goat anti-human IgM enzyme conjugate (■, diluted 1:500), goat anti-human IgG enzyme conjugate (○, diluted 1:1,000). *R. typhi* Renografin-purified antigen no. 3 diluted 1:250. (A) Human serum no. 1. (B) Human serum no. 2b 7/21/76.

cro-titer plate procedure. Since only a small portion of the antigen actually binds to the tubes, it should be possible to reuse the same antigen suspension for more than one tube or one well. In one instance we have coated tubes three times with the same antigen without loss of efficacy.

The ELISA employed here showed a variable amount of species specificity toward *R. typhi* and *R. prowazekii*, depending chiefly on the animal source of the antiserum, which decreased in the following order: mouse, guinea pig, human, and rabbit. This finding paralleled more closely the results obtained with the MA test using particulate antigens than the non-specific titers obtained with the CF test using the same group-specific antigens. As Reiss-Gutfreund et al. (24) have pointed out, the presence of type-specific components in the soluble group antigen has been suggested by a number of workers. Why the ELISA is more sensitive to these differences than the CF test is not known. Possibly, the linear OD scale employed in the ELISA can more readily detect twofold differences in serum titers than the CF microtiter test. In addition, the ELISA and CF tests may measure different types of immunoglobulins.

Ethanedithiol treatments of serum are clearly useful for distinguishing IgM and IgG antibodies in the CF and MA tests (17-19). The fact that this treatment is compatible with the ELISA permits one to check the specificity of commercial sources of anti-IgM or anti-IgG under test conditions or to perform a differential test if only anti-immunoglobulin conjugate is available. Furthermore, the ELISA is superior to the MA and CF tests for the detection of IgM and IgG, since titers are obtained directly rather than on a background of IgG, as is done in the latter two tests. Consequently, the sequential titrations of IgM and IgG antibodies in primary typhus infection, vaccination, or recrudescence of typhus (Brill-Zinsser disease) is greatly facilitated.

Although indirect fluorescent antibody (IFA) and indirect hemagglutination (IHA) tests have not been done here, recent results of Shirai et al. (29) and Philip et al. (23) suggest that the ELISA, as described here, is at least as sensitive as those procedures. For example, calculating from the data of Shirai et al. (29), the average of the ratios of IHA/CF and IFA/CF titers of 31 human typhus sera with positive CF titers against *R. typhi* antigens were 18 and 29, respectively, whereas our 7 human sera had average ELISA/CF titer ratios against *R. typhi* PR antigen of 113 with IgG conjugate. Similarly, microimmunofluorescence (MIF) test data on human serum against *R. prowazekii*

antigens had average titer ratios of 107 for 19 sera tested with anti-immunoglobulin conjugate (23), whereas the average ELISA/CF titer ratio against *R. prowazekii* PR antigen determined here was 125 with anti-IgG. In the case of infections with animal parasites, the ELISA was at least as sensitive as IFA or IHA (5, 25, 26). Since both ELISA and IFA require conjugates, but the former can be done without an expensive fluorescent microscope and subjective analysis, the ELISA is highly suitable as an alternative sensitive serological test. Furthermore, the ELISA is more flexible in the types of antigens that can be used than are the IFA or IHA techniques.

In summary, the ELISA procedure described here is a sensitive method for the detection of typhus-group antibodies in human and animal sera and shows some species specificity with soluble antigens. Renografin-purified rickettsiae are clearly superior as a source of antigen compared with the standard antigens prepared from infected yolk sacs. The ELISA procedure should prove to be a valuable adjunct to the rickettsial serology now available in clinical laboratories. It can also be a powerful research tool in studies of rickettsial antigens and the humoral response to rickettsial infection.

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