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Enzyme Immunoassay and Radioimmunoprecipitation Tests for the Detection of Antibodies to *Rochalimaea* (Rickettsia) *quintana* (39655)[1]

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Introduction. Epidemics of trench fever, caused by *Rochalimaea* (Rickettsia) *quintana*, occurred during the First and Second World Wars as well as the Spanish Civil War, and the disease remains endemic in some areas of the world. The infective agent can be isolated from the blood (1), and sensitive serological techniques are required for the detection of specific antibodies. Complement fixation tests (CF) with whole cell or soluble cytoplasmic antigens detect *R. quintana* antibodies (2), but lack of sensitivity has led to introduction of immunofluorescent (IF) and passive hemagglutination (PHA) techniques. However, in a seroepidemiological survey in an area suspected of endemicity for trench fever, only 33% of the people were positive by either IF or PHA tests, although the combined positives by both were 55% of the total sampled (unpublished data). In an attempt to improve sensitivity, a radioimmunoprecipitation test and an enzyme immunoassay test were developed and compared to IF and PHA for detecting *R. quintana* antibodies in serum.

Materials and methods. Soluble antigen. Cells of *R. quintana*, strain Fuller (3) or Guadalupe (4), were grown on hemolyzed horse blood agar for 5 days at 37° in a moist atmosphere of 5% CO_2. The cells were harvested in a sucrose-phosphate-glutamate solution (5), washed three times, resuspended in 0.1 *M* phosphate-buffered saline (PBS), pH 7.2, and disrupted by sonication for 30 min at 1.5 A (Model 60 W Ultrasonic Disintegrator, M.S.E., London). After centrifugation at 75,000*g* for 1 hr, the supernatant fluid served as the soluble antigen. The antigen was stable at 4° for several months with added thiomersol at a 1:10,000 final concentration. Protein concentration was estimated by use of the Folin-Ciocalteau reagent (6).

Sera. Animal sera. After collecting preimmunization sera, animals were inoculated with soluble or whole cell antigen. Equal 1-ml vol of soluble antigen from *R. quintana* (containing 300-500 μg of protein/ml) and Freund's complete adjuvant were mixed and inoculated sc into New Zealand white rabbits and Hartley strain guinea pigs. Rabbits were bled at 3 and 4 weeks. After 6 weeks, guinea pigs were given two further inoculations sc; serum samples were taken eight weeks after the first inoculation. For guinea pig antiserum to *R. quintana* (Fuller) whole cells, equal 1-ml vol of washed cells and Freund's complete adjuvant were mixed and inoculated sc again 6 weeks later. Serum samples were taken 8 weeks after the first inoculation.

Human sera. Sera were obtained from residents of the Nabeur region of Tunisia, an area suspected of being endemic for trench fever. Sera were also obtained from Boston area laboratory personnel.

Passive hemagglutination. Tanned sheep red blood cells (SRBC) were used in a standard method (7). SRBC were coated with *R. quintana* (Fuller) soluble antigen at 5 to 10 μg of protein per ml of SRBC. These were added to sera previously inactivated (56° for 30 min) and adsorbed with SRBC.

Immunofluorescence techniques. The indirect method was used. *R. quintana* whole cells were suspended in a solution of 5% (w/v) normal yolk sac in PBS (homogenized by use of a Waring blender) and fixed on glass slides with acetone. Test or control sera were added and incubated 30 min at 37°. Fluorescein isothiocyanate-conjugated antiglobulin (Difco Laboratores, Detroit, Mich.) was added and incubated for 30 min at 37°. The slides were washed with PBS,

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mounted in glycerol, and observed under a Zeiss fluorescence microscope equipped with BG12 and BG38 exciter filters and an OG1 barrier filter.

Radioactive antigen. Soluble antigen of *R. quintana* (Fuller) was labeled with ^{125}I by use of the chloramine T method (8). $Na^{125}I$ (New England Nuclear, Boston, Mass.) was diluted with 0.5 *M* phosphate buffer, pH 7.5 to contain 10 mCi/ml. One millicurie (0.1 ml) was added to 0.4 ml of antigen (containing 1.2 mg/ml of protein) plus 1.1 ml of 0.05 *M* phosphate buffer, pH 7.4. Chloramine T (500 μg in 0.1 ml) was added, and the reactants were stirred at room temperature for 30 sec. Sodium metabisulphite (1 mg in 0.1 ml) plus 1 mg (0.1 ml) of KI was added to stop the reaction. Labeled material was partially purified by filtration on Sephadex G-50. Fractions were collected and assayed for radioactivity by counting ^{125}I disintegration in a Nuclear-Chicago automatic gamma counter. The labeled fractions were pooled and dialyzed for 48 hr against PBS to remove residual iodine. Precipitation with 5% trichloracetic acid showed that approximately 97% of the ^{125}I was protein associated. The sp act of the antigen preparations was 20 to 30 μCi/mg of protein.

Radioimmunoprecipitation test (RIP). The procedure was similar to those described by Gerloff *et al.* (9), Dalrymple *et al.* (10), and others. ^{125}I-Labeled antigen and sera were diluted in borate-buffered saline (BSB), pH 8.0; each serum was tested in triplicate. A 0.2-ml vol of test serum plus 0.1 ml of labeled antigen were incubated in plastic tubes at 37° for 1 hr. Anti-globulin (0.1 ml) was added, and the mixtures were incubated at 37° for 1 hr, followed by incubation at 4° for 18 hr. Controls consisted of normal serum plus labeled antigen plus anti-globulin; labeled antigen plus anti-globulin; and labeled antigen plus BSB plus anti-globulin. Test and control preparations were centrifuged at 4000*g* for 30 min. Samples (100 μl) of the supernatant fluids were assayed for radioactivity (cpm). The precipitates were washed three times with BSB by centrifugation and also counted. Binding of antigen, and hence, a positive serum, was calculated in two ways: (i) by the difference in the radioactivity (cpm) of washed precipitates of test and control samples, and (ii) by the percentage of radioactivity in the supernatant fluids, as described by Brunner and Chanock (11); $[(S_c - S_t)/S_c] \times 100 = X\%$, where S_t is the mean counts per minute in supernatant fluids from a test serum, and S_c the mean counts per minute in control supernatant fluids.

Enzyme-labeled anti-globulin. Globulin fractions of goat anti-human IgG and goat anti-rabbit IgG (Antibodies, Inc., Davis, Calif.) were obtained by $(NH_4)_2SO_4$ precipitation (40% saturation) of whole sera. The method for coupling the globulins with enzyme (horseradish peroxidase, type IV, Worthington Biochemical Corp., Freehold, N.J.) was by use of periodate (12). Peroxidase-labeled globulin was separated from unlabeled material by gel-filtration on 2.5 x 80-cm columns of Sephadex G-200. Fractions that gave maximum adsorption in a spectrophotometer at both 403 (enzyme) and 280 nm (protein) were pooled and precipitated by addition of $(NH_4)_2SO_4$ to 50% saturation. The precipitate was suspended in a volume of PBS to give a protein concentration of approximately 2 mg/ml and dialyzed against PBS. The preparations were tested for immunologic reactivity in gel-diffusion plates against the appropriate globulin and stored at −4° until used.

Enzyme immunoassay. The procedure for solid-phase enzyme immunoassay (EIA) was based on the enzyme-linked immunosorbent assay of Engvall and Perlmann (13), as modified by Ruitenberg *et al.* (14). Soluble *R. quintana* antigen at 100 μg/ml in 0.1 *M* sodium carbonate buffer, pH 9.5, was added to round-bottom wells in polystyrene microtiter plates (Cooke Engineering, Alexandria, Va.), 0.2 ml/well. The antigen was absorbed 3 hr at 37°, plus 4 days at 4°. The antigen was removed, and 0.3 ml/well of carbonate buffer plus 1% (w/v) bovine serum albumin (BSA) was added.

The plates were incubated at 4° overnight and washed three times with distilled water plus 0.05% (v/v) Tween 20. Test and control sera, diluted in PBS, plus 0.05% (v/v) Tween 20 were added (0.05 ml/well) and incubated for 30 min at 37°. The plates were washed as above, and peroxidase-conju-

gated anti-globulin was added (0.05 ml/well) at a 1:100 to 1:200 dilution. The diluent for the conjugates was PBS with 1% (w/v) BSA and 0.05% (v/v) Tween 20 added. The plates were incubated for 30 min at 37° and washed as above; 0.3 ml/well of substrate [0.05% 5-amino salicylic acid in distilled water plus 0.005% H_2O_2, Ref. (14)] was added. After 30–60 min at room temperature, the reaction was stopped with 0.1 ml of 1 *N* NaOH. A red-brown reaction product was formed; the end points were read visually by comparison with controls (antigen plus normal serum and antigen plus PBS).

Results. Animal sera. A comparison of the reactivity of hyperimmune animal sera in IF, PHA, RIP, and EIA is shown in Table I. By the third week after inoculation with soluble antigen, rabbit sera gave similar titers in IF and PHA and slightly higher titers in RIP and EIA. No differences were seen between rabbit sera prepared against Fuller or Guadalupe strains of *R. quintana*. Higher titers were demonstrated in guinea pigs which had received multiple inoculations. No differences were noted between guinea pig antisera prepared with whole cells and those with soluble antigen, nor did use of whole cells produce higher antibody titers than soluble antigen. Titers of preimmunization sera were <1:10 in all tests.

Human sera. Results with 10 sera are shown in Table II. Of these sera, six were positive in IF tests, and seven were positive in PHA tests. However, all 10 sera were positive in both RIP and EIA tests, with generally higher titers. Sera obtained from four other people in the same region of Tunisia were negative by all four tests (titers <1:10), as were two sera obtained locally from laboratory personnel. All sera were examined twice by RIP and EIA on different days: The variation was never more than one twofold dilution.

Discussion. Of 14 human sera (Tunisian) tested, totals of six and seven were positive in IF and PHA, respectively, whereas 10 sera reacted in both RIP and EIA. This indicates an increased sensitivity of these tests, even though individual titers of some positive sera may be the same or higher in PHA or IF. Although RIP and EIA each detected an equal number of positive sera, the advantages of EIA are ease of reagent preparation, simplicity and safety of use, and stability of label.

Most EIA techniques use plastic tubes rather than microtiter plates. The tubes allow for the easy reading of reactions spectrophotometrically, whereas plates require lower volumes of reagents. The latter is important if high concentrations of antigen are needed for optimal results and the supply is limited. Although some investigators have found that as little as 5 μg/ml of antigen will give a successful EIA test (14), and it is known that, for some proteins, concentrations above 10 μg/ml result in a decreased percentage of adsorption (15), we found

TABLE I. Reactivity of Animal Sera in Immunofluorescence (IF), Passive Hemagglutination (PHA), Radioimmunoprecipitation (RIP), and Enzyme Immunoassay (EIA) Tests.

Animal	No.	Antigen	*R. quintana* strain[a]	Weeks post-inoculation	Antibody titer[b]			
					IF	PHA	RIP	EIA
Rabbit	1	Soluble	G	3	640	320	1,280	2,560
				4	2,560	5,000	5,000	10,000
	2	Soluble	G	3	640	640	1,280	1,280
				4	640	640	640	1,280
	3	Soluble	F	4	640	160	1,280	2,560
Guinea pig	1	Whole cell	F	8	2,560	2,560	5,120	n.t.[c]
	2	Soluble	F	8	10,000	20,000	20,000	
	3	Soluble	F	8	10,000	20,000	20,000	
	4	Soluble	F	8	2,560	1,280	2,560	

[a] Guadalupe (G), Fuller (F).
[b] Reciprocal of serum dilution.
[c] Not tested.

TABLE II. REACTIVITY OF HUMAN SERA TO *R. QUINTANA* ANTIGEN IN IMMUNOFLUORESCENT (IF), PASSIVE HEMAGGLUTINATION (PHA), RADIOIMMUNOPRECIPITATION (RIP), AND ENZYME IMMUNOASSAY (EIA) TESTS.

Serum No.	Antibody titer[a]			
	IF	PHA	RIP	EIA
703	<10	320	320	160
707	20	20	40	40
708	<10	80	80	80
714	<10	160	160	160
721	10	80	80	40
739	<10	80	80	160
753	40	<10	20	40
754	20	<10	20	40
778	40	160	320	160
785	160	<10	40	40

[a] Reciprocal of serum dilution.

that concentrations of 100 μg/ml of protein of *R. quintana* antigen were needed. Thus, the microtiter assay is more suitable in this instance.

The sensitivity of the EIA test assures accurate serodiagnosis of *R. quintana* infection and its simplicity should expedite delineation of the geographical distribution of trench fever. The method may be applicable to other rickettsial infections as well.

Summary. Solid-phase enzyme immunoassay (EIA) and radioimmunoprecipitation tests were developed for detection of antibodies to *Rochalimaea* (Rickettsia) *quintana* in trench fever. Both were equally sensitive, and both detected more positive sera than either passive hemagglutination or immunofluorescence tests. The advantages of EIA are the stability of label and ease and safety of use.

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