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Immunological Properties of *Rickettsia rickettsii* Purified by Zonal Centrifugation

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The properties of *Rickettsia rickettsii* purified from infected chicken yolk sacs or mouse L cell cultures by sucrose density gradient centrifugation in a zonal rotor were examined in various ways. Rickettsiae derived from both L cells and yolk sacs had similar compositions: about 12% nitrogen, 1.5% phosphorus, 5% carbohydrate, and 30% fatty acids. On a dry-weight basis, purified rickettsiae were at least 2,000 times as effective as a commercial spotted fever vaccine in protecting guinea pigs against infection with spotted fever rickettsiae and mice against death from toxin of *R. rickettsii*. Gradient-purified rickettsiae (0.6 μg) induced a serological response in guinea pigs of the same magnitude as that stimulated by 1,600 μg of the commercial vaccine. Gradient-purified rickettsiae had little group reactivity in complement fixation tests but became anti-complementary upon storage. Microagglutination and hemagglutination tests with the purified antigen gave promise of usefulness in diagnosis of spotted fever. These results suggest that zonal centrifugation may be a valuable procedure for the preparation of *R. rickettsii* vaccine and diagnostic reagent.

Recently we reported that the spotted fever rickettsia, *Rickettsia rickettsii*, could be obtained in a high state of purity from infected yolk sacs of chicken embryos or L cell tissue cultures by centrifugation in a linear sucrose gradient in a zonal rotor (1). Since the effects of such treatment on the biological properties were unknown, a study was undertaken to determine how well vaccination with purified rickettsiae would protect animals against challenge with virulent spotted fever rickettsiae or their toxin. Also, usefulness of the purified rickettsiae as a diagnostic reagent in various serological tests was investigated. In addition, the basic chemical composition of the rickettsiae was determined. Results of these studies are summarized in the following report.

MATERIALS AND METHODS

Animals. Approximately 400-g male Hartley strain guinea pigs, obtained locally, and 3- to 4-week-old male white mice of the Rocky Mountain Laboratory (RML) strain were used for our experiments.

Strains of rickettsiae. Egg-adapted *R. rickettsii* strain R, isolated from *Dermacentor andersoni* ticks collected in the Bitter Root Valley of western Montana, was used in the preparation of gradient-purified rickettsiae and "conventional" complement-fixing (CF) antigen by ether extraction of infected yolk sacs (see below) and as the challenge organism in toxicity tests and all but one protection test. The Sawtooth strain of *R. rickettsii*, also isolated from *D. andersoni* ticks collected in the Bitter Root Valley and since maintained in ticks, was used in the preparation of antiserum for serological tests. The Sheila Smith strain of *R. rickettsii* was utilized by our commercial source of vaccine and as the challenge organism in one of our protection tests. This strain was obtained from a patient in Missoula, Mont.

Rickettsiae of other species used for the preparation of antisera were the Simko strain of *R. conorii*, *R. australis* strain 32, *R. akari* strain 7, the Breinl strain of *R. prowazekii*, and California strain 805 of *Coxiella burnetii* in phase I. These strains have been maintained in eggs or ticks at RML for at least several years.

Antisera. Anti-rickettsial sera were prepared from blood collected from convalescent guinea pigs infected with yolk sac or tick suspensions containing the various rickettsiae. Antiserum to *R. rickettsii*, devoid of antibody to yolk sac antigens, was obtained 37 days after injection of a suspension of ticks containing infectious rickettsiae of the Sawtooth strain. There was no significant difference in the behavior of rickettsiae of the R and Sawtooth strains in CF tests. Guinea pig antiserum to *C. burnetii* was collected 3 weeks after infection, which followed subcutaneous inoculation with purified, ether-inactivated, Q fever rickettsiae by 3 weeks. Animals infected with *R. akari* and *R. conorii* were bled 1 month later, and those infected with *R. prowazekii* were bled 3 weeks later. *R. australis* antiserum was obtained from guinea pigs 2 weeks after the last of six weekly inoculations of living rickettsiae. Preparation of the anti-yolk sac serum was described previously (1).

Cultivation of R. rickettsii for zonal

centrifugation. The R strain was cultivated either in embryonated chicken eggs according to the method of Stoenner et al. (21) or in L cells (1).

Commercial vaccine. A vaccine prepared by the method of Cox (7) at Lederle Laboratories, Pearl River, N.Y., from chicken embryo yolk sacs infected with the Sheila Smith strain of *R. rickettsii* was used, before the expiration date, as a control in some of our experiments. To estimate the dry weight of this vaccine, 3 ml of the vaccine was dialyzed against distilled water and lyophilized. The lyophilized material was not used in any of the bioassays.

Density gradient zonal centrifugation. Centrifugation procedures were essentially the same as those reported earlier (1). Some of the samples used in the present study were centrifuged in a Beckman JCF-Z rotor equipped with the zonal core. The rotor was loaded while spinning at 2,000 rpm (20 C) with 1,500 ml of a 10 to 55% linear sucrose gradient in 0.07 M phosphate-buffered saline (PBS; pH 6.0) followed by 450 ml of 55% sucrose in PBS as a cushion. One hundred milliliters of sample and then 50 ml of distilled water as an overlay were injected into the center line leading to the top of the gradient. The rotor was spun at 10,000 rpm for 30 min at 20 C and then slowed to 2,000 rpm for unloading. The subsequent steps of analysis and washing were the same as described earlier (1). Differences in properties of rickettsiae purified with different rotors were not detected. Either sodium azide (0.02%) or merthiolate (1:10,000) was added as a preservative.

Serological tests. The CF test was the microtiter test described by Lennette (16). Two kinds of preparations were used as antigen: gradient-purified *R. rickettsii*, and *R. rickettsii*-infected yolk sac suspensions extracted with ether ("conventional" antigen). The method of ether treatment used by the Serology Department of RML was adapted from that described by Topping and Shepard (22). Infected yolk sacs were blended for about 2 min with 5 volumes of chilled 0.07 M phosphate buffer, pH 5.8. Formalin was added to a concentration of 0.2%, and the preparation was held at 4 C, with occasional shaking, for about 16 h. Two volumes of diethyl ether was added and the preparation was again incubated at 4 C with occasional shaking. After about 24 h the aqueous phase, containing both particulate and soluble antigens, was drawn off and used as antigen. Two units of antigen, as determined by cross-box titrations against homologous antisera, was used in testing all other sera.

Gradient-purified rickettsiae were also used as antigen in the microagglutination (MA) test of Fiset et al. (11). After overnight incubation, 0.025 ml of aqueous acridine orange (1:5,000) was added to each well to make the rickettsiae more readily visible.

The hemagglutination (HA) procedure described by Chang (6) for typhus rickettsiae was adapted for use with spotted fever rickettsiae. For our study, gradient-purified lyophilized rickettsiae were suspended in saline (1 mg/ml); the suspension was made 0.2 N with respect to NaOH, boiled for 30 min, and then dialyzed against Chang's buffer. This material was used to sensitize sheep erythrocytes. Since antisera to *R. rickettsii* at low dilutions sometimes also agglutinated normal sheep erythrocytes, each serum was tested against both sensitized and normal sheep erythrocytes in microtiter plates. Antibody responses were considered negative unless sensitized erythrocytes were agglutinated by at least a twofold higher serum dilution than were normal erythrocytes.

Bioassays. Immunogenicity of several rickettsial antigens in guinea pigs was determined by a modification of the procedure of Bell and Stoenner (5). Guinea pigs in groups of six to eight were inoculated intraperitoneally with graded doses of vaccine diluted in 0.07 M PBS (pH 7.4) and challenged intraperitoneally 10 days later with 10,000 guinea pig 50% infective doses of a standardized suspension of yolk sac-grown *R. rickettsii*, usually the R strain but in one experiment the Sheila Smith strain. Rectal temperatures were recorded daily for at least 12 days and areas under the fever curves, with fever defined as temperature above 39.6 C, were measured. The 50% protective dose (PD_{50}), the dose of vaccine which reduced the febrile response to half that observed in control animals, was then determined graphically.

The mouse protection test for potency assay of the gradient-purified rickettsiae and the commercial vaccine was also used (3, 5). Three-week-old mice were inoculated intraperitoneally with 0.5-ml volumes of graded doses of the preparations in PBS. Seven days later the mice were inoculated intravenously with a yolk sac suspension containing 2 50% lethal doses (LD_{50}) of *R. rickettsii* toxin. (The LD_{50} dose was determined in 28-day-old normal mice.) Deaths were recorded at 24 h, and the dose protecting 50% of the mice from the lethal effects of the toxin was calculated by the method of Reed and Muench (20).

Chemical analyses. Nitrogen was determined by the procedure of Johnson (14). Total carbohydrate was estimated by the tryptophan method (8), using glucose as a standard. Phosphorus was determined colorimetrically after digestion with sulfuric acid and hydrogen peroxide (9), and esterified fatty acids and amide-linked fatty acids, with palmitic acid as a standard, were determined by the method of Haskins (12). Total protein released from the rickettsiae heated to 100 C with NaOH was determined colorimetrically after reduction of the Folin-Ciocalteau reagent (13). Bovine serum albumin was used as the standard.

RESULTS

Chemical composition. Results of chemical analyses of rickettsial preparations from either infected L cells or yolk sacs are presented in Table 1. Regardless of the kind of tissue used for cultivation, the compositions of the lots were similar. Nitrogen content averaged 12.1%, phosphorus 1.42%, total carbohydrate 5.25%, fatty acids 30.7%, and total protein 66.3%.

Diagnostic reagent. The suitability of *R. rickettsii* purified by density gradient zonal centrifugation as antigen in serological tests was investigated.

Hemagglutinin and agglutinin titers of sera obtained from guinea pigs 3 weeks after infection with *R. rickettsii* are presented in Table 2.

TABLE 1. *Chemical composition of Rickettsia rickettsii purified by sucrose density gradient zonal centrifugation from infected L cells or yolk sacs*

Preparation no.	Source	Nitrogen (%)	Phosphorus (%)	Total carbohydrate (%)	Fatty acid (%)	Total protein (%)
1	L cells	11.9	1.35	5.57	31.1	65.4
2	L cells	12.0	1.58	4.62	27.8	63.7
3	Yolk sacs	12.4	1.32	5.43	33.2	68.9
4	Yolk sacs	12.2	1.43	5.36	—[a]	67.1

[a] Insufficient sample available.

TABLE 2. *Antibody titers of sera from guinea pigs infected with Rickettsia rickettsii*

Guinea pig[a]	Test		
	Hemagglutination[b]	Microagglutination[b]	Complement fixation[c]
1	256[d]	1,024	96
2	512	4,096	128
3	1,024	128	128
4	256	2,048	128
5	64	2,048	128
6	64	512	64
7	512	2,048	384
8	1,024	1,024	256

[a] Sera obtained 21 days after intraperitoneal inoculation of 10,000 50% infective doses of *R. rickettsii*.

[b] Antigen purified by sucrose density gradient zonal centrifugation of suspensions of *R. rickettsii*-infected L cells.

[c] Antigen consisted of the aqueous phase of a *R. rickettsii*-infected yolk sac suspension extracted with ether.

[d] Reciprocal of serum dilution.

For comparison, CF titers from a test using the conventional, ether-extracted yolk sac antigen are included. Antibody was detected in all sera with each of the tests; in general, higher titers were observed with the HA and MA tests. None of the antigens reacted with antisera to *C. burnetii* or *R. prowazekii*.

In addition, the gradient-purified antigen was compared with the conventional yolk sac antigen in CF tests of 31 human sera sent by physicians to RML for serological tests and also of several heterologous guinea pig sera. These sera had been frozen for 2 to 17 months before the tests. A variety of responses was observed among the human sera, and representative results are presented in Table 3. Three of the 31 sera, including no. 1, were negative in all tests; three other sera, as no. 6, were positive in all tests. One serum (no. 3) reacted only in the CF test using the ether-extracted yolk sac suspension. Ten sera, like no. 5, were positive in the HA test only, and five sera, including no. 2, were positive in all tests except the CF test using gradient-purified *R. rickettsii*. Six sera, as no. 4, reacted only in the two agglutination tests, and three sera, including no. 7, reacted only in the CF test using the ether-extracted antigen and in the HA test.

Guinea pig sera reacted more uniformly in the various tests. The specific antiserum (no. 8) had similar titers in all tests, and the anti-*C. burnetii* and *R. prowazekii* sera did not cross-react with the *R. rickettsii* antigens. Yolk sac antigen was detected only in the ether-extracted yolk sac suspension.

To determine whether the gradient-purified rickettsiae still possessed CF group antigen, the purified rickettsiae, as well as the conventional yolk sac antigen, were tested against guinea pig antisera to several rickettsiae of the spotted fever group. Compared with conventional yolk sac antigen, the content of the CF group antigen on the purified rickettsiae was considerably reduced (Table 4).

Some information relating to stability of the gradient-purified antigens was obtained. Preparations stored at 4 C in saline containing merthiolate (1:10,000) performed well in the CF test when freshly prepared, but 2 months later these preparations were too anti-complementary to use. In contrast, the same type of antigen appeared unchanged in MA tests when last tested 3 months after preparation. Antigen prepared for the HA test was not affected by storage at 4 C for 9 months.

Antibody response of guinea pigs. The antibody responses of five to six guinea pigs inoculated with single doses of either 1,600 μg (dry weight) of the commercial vaccine or 0.6 μg (dry weight) of gradient-purified *R. rickettsii* were measured by CF, MA, and HA tests. Fourfold lower doses of the antigens were used in an earlier experiment, but the levels of antibody detected were even lower than those reported here. Agglutinin titers in sera from animals inoculated with either antigen reached a peak at about 5 days and then quickly dropped (Fig. 1). HA and CF titers reached their peaks in 10 to 20

TABLE 3. *Antibody titers of various stored sera in tests using antigens of Rickettsia rickettsii*

Serum[a]	Test			
	Complement fixation		Microagglutination (gradient purified)	Hemagglutination (gradient purified)
	Ether-extracted yolk-sac suspension[b]	Gradient purified		
1. Human (MS) [1][c]	0[d]	0	0	0
2. Human (MS) [31]	48[e]	0	128	≧1,024
3. Human (JL) [23]	24	0	0	0
4. Human (BR) [4]	0	0	16	32
5. Human (JD) [5]	0	0	0	32
6. Human (JS) [62]	48	32	32	128
7. Human (CC) [?]	4	0	0	32
8. Guinea pig anti-*R. rickettsii*	256	256	256	128
9. Guinea pig anti-*C. burnetii*	0	0	0	0
10. Guinea pig anti-*R. prowazekii*	0	0	0	0
11. Guinea pig anti-yolk sac	24	0	0	0

[a] Human sera stored at −12 C 2 to 17 months before test.
[b] Type of *R. rickettsii* preparation.
[c] Numbers in brackets indicate days since onset of clinical symptoms.
[d] For complement fixation tests, 0 = negative at 1:8 serum dilution; for microagglutination and hemagglutination tests, 0 = negative at 1:4.
[e] Reciprocal of serum dilution.

TABLE 4. *Antibody titers of antisera to rickettsiae in the spotted fever group in complement fixation tests using antigens of Rickettsia rickettsii*

Antiserum[a]	Type of *R. rickettsii* antigen	
	Ether-extracted yolk-sac suspension	Gradient purified
Rickettsia rickettsii	192[b]	128
R. akari	32	8
R. conorii	32	2
R. australis	96	12

[a] Antisera at a dilution of 1:8 did not react with ether-extracted yolk sac suspensions of *Coxiella burnetii* and *R. prowazekii*, rickettsiae not included in the Rocky Mountain spotted fever group.
[b] Reciprocal of serum dilution.

days and then dropped off more slowly. For the doses used, responses to the gradient-purified antigen were somewhat greater than to the commercial vaccine.

Immunogenicity. The quantities of *R. rickettsii* purified from both yolk sacs and L cells required to protect guinea pigs against infection with spotted fever rickettsiae are presented in Table 5. Purified rickettsiae in aqueous suspension protected best (no. 2 and 4); PD_{50} values for yolk sac and L cell-cultivated rickettsiae were 3 to 4 logs lower than that for the commercial vaccine (no. 5). For reasons not yet clear, lyophilization adversely affected protective potency, but the lyophilized preparations (no. 1 and 3), on a weight basis, were still at least 2 logs better than the commercial vaccine (no. 5).

Since the commercial vaccine used in the above experiment was prepared from the Sheila Smith strain and rickettsiae of the R strain were used for challenge, PD_{50} values for gradient-purified rickettsiae (aqueous suspension of L cell-grown rickettsiae) and the commercial vaccine in guinea pigs challenged with the Sheila Smith strain were determined. Values of 0.0081 and 82.7 μg, respectively, were obtained, which indicated that the difference in potency of the two preparations was probably not related to the difference in strains used for challenge.

A similar relationship was observed in experiments in which mice vaccinated with graded doses of the various preparations were challenged with 2 LD_{50} of toxin of *R. rickettsii* (Table 6). Aqueous suspensions of gradient-purified rickettsiae (no. 2 and 4) were about 5,000 times more protective than was the commercial vaccine (no. 5), and the lyophilized preparations (no. 1 and 3) were about 25 to 45 times more potent.

DISCUSSION

Results from the various studies of *R. rickettsii* purified by density gradient zonal centrif-

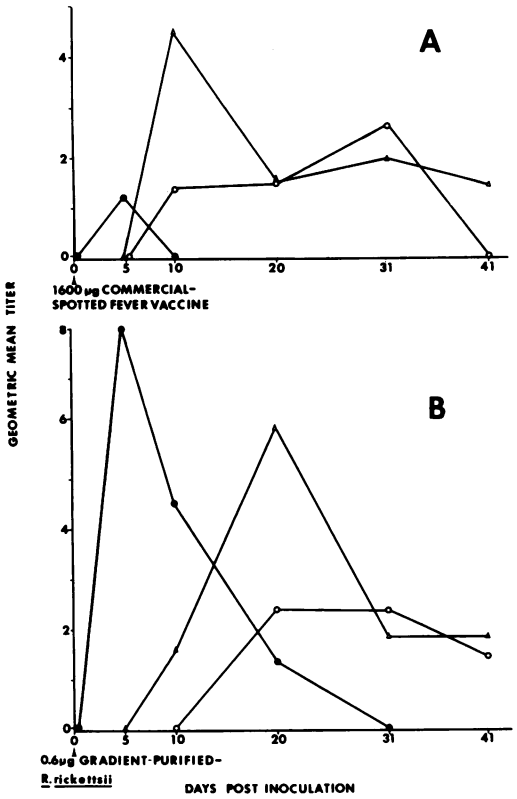

FIG. 1. *Antibody response of five to six guinea pigs inoculated intraperitoneally with either commercial spotted fever vaccine (A) or gradient-purified R. rickettsii (B). Symbols: (●) Microagglutination test; (△) hemagglutination test; (○) complement fixation test.*

ugation indicate that the purification procedure did not adversely affect the properties of the organisms. Indeed, potency of the purified rickettsiae in the bioassays was very high. Also, gross chemical composition of the purified rickettsiae was similar to that reported for other rickettsiae (2, 17, 18, 23).

Compared with the presently available commercial vaccine, the gradient-purified rickettsiae were extremely potent in our bioassays. On a dry-weight basis, 2,000 to 6,000 times more commercial vaccine than gradient-purified rickettsiae was required to provide the same degree of protection to guinea pigs against infection with spotted fever rickettsiae. Similar quantities of each preparation protected mice against the lethal effects of the toxin of *R. rickettsii*. Possible reasons for the disparity in potency are (i) contamination of the commercial vaccine with tissue from the chicken embryo, (ii) inactivation of *R. rickettsii* immunogen(s) during preparation of the commercial vaccine, and (iii) inactivation of *R. rickettsii* immunogen(s) in commercial vaccine during storage.

It is unfortunate that our stock of the Sheila Smith strain of *R. rickettsii* was not well adapted to eggs so that we could conveniently use that strain for our gradient-purified rickettsiae and toxin, because questions may arise regarding the validity of our comparisons of results of tests in which animals were inoculated with either purified rickettsiae of the R strain or commercial vaccine derived from the Sheila Smith strain and subsequently tested with the R strain only. However, certain considerations may make these comparisons more acceptable.

In the case of vaccine potency, we demonstrated that gradient-purified rickettsiae of the R strain were clearly as superior to the commercial vaccine in guinea pigs challenged with the Sheila Smith strain as in those challenged with the R strain. Also, Bell and Stoenner (4) showed that three pathogenic strains of *R. rickettsii*

TABLE 5. *Protection of guinea pigs by formalin-inactivated gradient-purified Rickettsia rickettsii against infection with R. rickettsii*

Prepn no.	Purified from:	Storage state	PD_{50}[a] (μg)
1	Yolk sacs	Lyophilized	0.23
2	Yolk sacs	Aqueous suspension	0.0054
3	L cells	Lyophilized	0.19
4	L cells	Aqueous suspension	0.017
5[b]	Yolk sacs	Aqueous suspension	34.8

[a] Dose which reduced the febrile response to half that observed in control animals.
[b] Commercial vaccine.

TABLE 6. *Protection of mice by formalin-inactivated gradient-purified Rickettsia rickettsii against toxin of R. rickettsii*

Prepn no.	Purified from:	Storage state	PD_{50}[a] (μg)
1	Yolk sacs	Lyophilized	0.28
2	Yolk sacs	Aqueous suspension	0.0024
3	L cells	Lyophilized	0.52
4	L cells	Aqueous suspension	0.0022
5[b]	Yolk sacs	Aqueous suspension	13.0

[a] Dose which protected 50% of the mice from death after challenge with 2 LD_{50} of toxin of *R. rickettsii*.
[b] Commercial vaccine.

from widely diverse sources could not be differentiated by toxin neutralization tests. Finally, after the above experiments were completed, CF tests with ether-extracted yolk sac suspensions from eggs infected with either the R or Sheila Smith strain gave similar antibody titers for sera from guinea pigs infected with the two strains. In view of these similar properties of the two strains, it seems probable that our results would not have differed significantly if the Sheila Smith strain rather than the R strain had been used exclusively in our experiments.

Two other studies concerning spotted fever vaccine potency have appeared recently. Kenyon et al. (15) reported that a vaccine made from *R. rickettsii* grown in primary cultures of cells from duck embryos was more than 900 times as active as a commercial yolk sac vaccine in protecting guinea pigs against challenge with *R. rickettsii*. The reason for the greater potency was not definitely established, but they reported that duck embryo cells contained many more rickettsiae than did yolk sac cells, despite a similarity in infectivity for embryonated eggs. Since our results demonstrated that rickettsiae purified from yolk sacs by density gradient zonal centrifugation were highly immunogenic, we would agree with their suggestion that the reason for the superiority of the duck embryo cell vaccine over the yolk sac vaccine is due in part to the greater number of rickettsiae present in infected duck embryo cells rather than to a greater inherent immunogenicity per rickettsial cell.

In the second study, DuPont et al. (10) reported that commercial vaccine provided very limited protection to humans. Twelve of 13 volunteers who received commercial vaccine 3 to 6 months before intradermal challenge with *R. rickettsii* developed clinical spotted fever. However, compared with unvaccinated controls the incubation period was lengthened and the duration of fever was decreased. No amelioration of disease was observed in two individuals vaccinated 16 months before challenge. In contrast, no symptoms of disease were noted in six individuals challenged 4 to 17 months after experimental infection. Although gradient-purified rickettsiae were clearly superior to commercial vaccine in our experiments with guinea pigs and mice, without field trials one cannot conclude that the purified rickettsiae would afford practical immunity to humans.

From our experience with two kinds of tests to evaluate the efficacy of spotted fever vaccines, we would recommend the mouse protection test over the guinea pig protection test. Similar values for the PD_{50} were obtained with both tests, as was noted earlier with ether-extracted yolk sac vaccines (5), but the mouse test was much more economical in terms of cost of animals, time, and space requirements. However, the ultimate animal model will be the one that can accurately predict the response of humans.

Results of serological tests with a limited number of human sera indicate that HA tests with gradient-purified rickettsiae may be of some value for diagnosis of spotted fever. Although antibody was detected earlier in vaccinated guinea pigs with the MA test than with the HA and CF tests, antibody was detected in patients' sera more often with the HA test than with any of the other serological tests, particularly during the early stages of illness. Possibly the failure of the purified rickettsiae to react with early CF antibody is due to the paucity of CF group antigen on the rickettsial surface. Others have indicated increased specificity of washed rickettsiae in CF tests (19).

Antibody was detected about as frequently with the MA test as with the CF test using conventional antigen, although agreement between tests was poor. If agglutinin and CF antibody responses of humans are similar to those of guinea pigs, the ability of MA and CF tests to detect antibody would depend in large part upon the length of time between infection and collection of the blood sample. Unfortunately, our information about the time periods involved for most of the serum samples is too incomplete to permit us to draw firmer conclusions. Obviously much additional testing is necessary to confirm the validity of these preliminary results.

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