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Indirect Hemagglutination Test for Detection of Antibody to *Rickettsia rickettsii* in Sera from Humans and Common Laboratory Animals

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Antibody production in humans and three species of laboratory animals infected with *Rickettsia rickettsii* was determined with the indirect hemagglutination test. Rabbits, guinea pigs, and mice were inoculated with *R. rickettsii* and bled at intervals. Antibody which agglutinated both fresh and glutaraldehyde-fixed sheep erythrocytes sensitized with antigen prepared either from purified rickettsiae or from infected yolk sacs was found in rabbit sera at all intervals tested (10 to 59 days postinfection). Antibody which agglutinated fresh but not glutaraldehyde-fixed erythrocytes sensitized with either of the above antigens was detected in guinea pig sera obtained 7, 14, and 28 days postinfection. Antibody was found in mice inoculated with 5.6×10^6 plaque-forming units of *R. rickettsii* but not in mice given 5.6×10^2 plaque-forming units. Peak indirect hemagglutination titers occurred in nonvaccinated human Rocky Mountain spotted fever patients about 3 weeks after onset of illness, and antibody was still detectable after 1 year. Both human immunoglobulin G and human immunoglobulin M antibodies agglutinated sensitized cells, but immunoglobulin M antibodies apparently were more efficient. The indirect hemagglutination test is useful for the titration of human, rabbit, guinea pig, and mouse antibodies when the appropriate erythrocytes are used.

During the past three decades there have been several studies of the indirect hemagglutination (IHA) test for detection of antibody to the Rocky Mountain spotted fever (RMSF) group of rickettsiae (1, 5, 12, 17, 21) as well as to other rickettsial agents (3, 4, 6, 7, 8, 10, 21, 23). Chang et al. (7) were the first to report that human group O erythrocytes, sensitized with substances prepared by alkali treatment of partially purified, ether-extracted *Rickettsia rickettsii*, were agglutinated by sera from patients as early as 6 days after onset of illness caused by certain agents of the RMSF group. This IHA test became positive before or at the same time as the complement fixation (CF) test did. IHA antibodies were detected in each of six rabbits but from only two of four guinea pigs inoculated with live rickettsiae; all of the animal sera were positive by the CF test.

Several years later Hersey et al. (12) compared levels of antibodies to murine typhus and RMSF rickettsiae determined by IHA and CF tests in sera from 85 patients. In contrast to the results of the previous study, these workers reported that antibodies were found in more sera and sometimes earlier with the CF test than with the IHA test. Increased sensitivity was noted for the CF test in some instances when 8 U of antigen was substituted for the usual 2 U of antigen.

More recent studies of sera from patients and animals support the potential usefulness of the IHA test first indicated by Chang et al. (7). Anacker et al. (1) found that the IHA test, adapted to microtiter plates and using an antigen prepared from *R. rickettsii* purified by sucrose density gradient centrifugation (2), was positive in a greater percentage of cases than was the CF test. In addition, the modified test detected antibody in eight of eight guinea pigs infected with *R. rickettsii*. Subsequently, in a comparison of serological methods for the diagnosis of RMSF, Philip et al. (17) reported that in a study of 324 sera submitted in 1974 to the North Carolina Division of Health Services, the IHA test, as well as the microimmunofluorescence (micro-IF) and microagglutination tests, was positive for RMSF about twice as often as was the CF test. Finally, Shirai et al. (21) introduced the use of glutaraldehyde-stabilized sheep erythrocytes so that the test could be performed under field conditions. Their results obtained with the treated erythrocytes were comparable to those from the indirect immunofluorescence test and

superior to those from the CF test. However, their test generally failed to detect antibodies in immune rat, guinea pig, and rabbit sera.

Although the above results indicate that the IHA test may be an additional valuable tool for the diagnosis and laboratory investigations of RMSF, our knowledge of IHA antibodies is incomplete. Little is known about the persistence and nature of the human immunoglobulins which agglutinate erythrocytes sensitized with rickettsial antigen. In addition, there is no explanation for the failure to detect antibodies in sera from infected animals with the IHA test in several instances. Therefore, we initiated the study described below to provide additional information about the antibody response of humans and to help resolve conflicting reports from several laboratories regarding the suitability of the IHA test for detection and titrations of antibodies to *R. rickettsii* in animal sera.

MATERIALS AND METHODS

Animals. Approximately 400-g male Hartley strain guinea pigs (obtained locally), 4-week-old male white mice of the Rocky Mountain Laboratory strain, and 2- to 3-kg New Zealand white rabbits of either sex (White's Wabbit Wanch, Kooskia, Idaho) were used for our experiments.

Antisera. Sera from four different species were tested: (i) human sera from RMSF patients, whose illnesses were confirmed by CF and micro-IF tests, (ii) sera from rabbits inoculated subcutaneously with 1.1×10^3 plaque-forming units of the R strain of *R. rickettsii* grown in yolk sacs of embryonated chicken eggs (22), (iii) sera from guinea pigs inoculated intraperitoneally with 6.3×10^3 plaque-forming units of *R. rickettsii*, and (iv) sera from mice inoculated intraperitoneally with either 5.6×10^2 or 5.6×10^6 plaque-forming units of *R. rickettsii*.

Serological tests. The IHA test used was basically that described by Chang et al. (7) and modified in our laboratory (1). Human group O and sheep erythrocytes were obtained locally and held in Alsever solution at 4°C. Some erythrocytes were treated with glutaraldehyde according to the method of Shirai et al. (21). Erthrocyte-sensitizing substance (ESS) was prepared from *R. rickettsii* grown in L cells and purified in a sucrose density gradient (2) or from a partially purified, ether-extracted suspension of yolk sac-grown *R. rickettsii* (Chang-type ESS) by sodium hydroxide digestion and dialysis against isotonic phosphate-saline solution (5). Antisera from a BCG-infected rabbit and from a guinea pig infected with the Breinl strain of *R. prowazekii* were used for negative control sera. In all instances reported below, the human sera or fractions were tested against unfixed human group O erythrocytes sensitized with ESS prepared from rickettsiae purified by density gradient centrifugation. All animal sera were tested against unfixed sheep erythrocytes sensitized with the same ESS; selected animal sera (to be described) were also tested against fixed and unfixed sheep erythrocytes sensitized with the Chang-type ESS.

The micro-IF test was performed according to the procedure of Philip et al. (18). Four fluorescein-labeled conjugates were used to detect the various immunoglobulin classes participating in the micro-IF reaction (14). One conjugate was broadly specific and detected both immunoglobulin G (IgG) and IgM antibodies to *R. rickettsii*, one was specific for IgG, one was specific for IgM, and one was specific for IgA. The CF test and the CF antigen prepared by ether extraction of *R. rickettsii*-infected yolk sacs were described previously (1).

Human IgG and IgM were separated from other immunoglobulins by centrifugation on 10 to 40% sucrose density gradients (9). The location of the globulins in the gradients was determined by immunodiffusion analyses with heavy-chain-specific anti-IgG, anti-IgA, and anti-IgM prepared in rabbits (Behring Diagnostics, Somerville, N.J.).

RESULTS

Persistence of *R. rickettsii* antibodies in humans. Sera taken from six RMSF patients at various intervals after infection were examined by the IHA and micro-IF tests (Table 1). In general, IHA titers were high during the early stages of infection and low but relatively stable after the first several months. The highest IHA titers occurred in nonvaccinated individuals, when, according to the micro-IF test, IgM antibodies were relatively prevalent and IgG antibodies were at low levels or undetectable. Antibodies were detected with the IHA test in sera from nonvaccinated patients for 1 year or more and in one serum from a vaccinated and infected individual (F) 3 years after onset of illness.

Immunoglobulin classes of human anti-*R. rickettsii* antibodies. Sera from two patients were fractionated on 10 to 40% sucrose density gradients to determine which classes of immunoglobulin were responsible for the agglutination of sensitized cells. Three pools of immunoglobulin were recovered after centrifugation: on the basis of immunodiffusion tests with heavy-chain-specific rabbit antisera, one pool contained a mixture of IgG and IgA, a second contained IgG only, and the third contained IgM only. Although results from the more sensitive micro-IF tests (Table 2) indicated that the separation of the various immunoglobulins was not as complete as was suggested by the immunodiffusion tests, an IgG fraction apparently free of IgM (patient 1) and an IgM fraction apparently free of IgG (patient 2) were obtained. Both of these immunoglobulins agglutinated sensitized erythrocytes (Table 2 and Fig. 1). Anti-rickettsial IgA antibody was not detected in these patients.

IHA antibody response of rabbits. Since other investigators were not able to consistently demonstrate *R. rickettsii* antibodies in the sera of rabbits, rats, and guinea pigs with the IHA

TABLE 1. *Persistence of R. rickettsii antibodies detected by IHA and micro-IF tests in sera from RMSF patients*

Patient	Vaccine history	Time since onset of disease	IHA titer	Micro-IF titer[a]		
				Immunoglobulin	IgM	IgG
A	None	17 days	≥4,096	512	2,048	0[b]
		89 days	512	64	64	Trace
		7 mo	128	32	16	32
		15 mo	32	32	0	16
B	None	3 days	32	0	0	0
		25 days	2,048	128	256	Trace
		105 days	512	64	64	32
		8 mo	256	32	16	16
		1 yr	256	32	16	16
C	None	24 days	1,024	64	64	32
		144 days	256	2,048	0	1,024
		10 mo	32	256	0	128
		14 mo	64	128	0	64
D	None	6 days	8	0	0	0
		24 days	2,048	4,096	8,192	512
		4 mo	512	32,768	Trace	16,384
E	3 doses 22 yr ago	6 days	128	512	256	512
		4 mo	128	1,024	256	256
		10 mo	256	512	128	256
		13 mo	128	256	128	256
F	Many doses	8 days	64	512	64	512
		60 days	256	8,192	2,048	4,096
		1 yr	64	512	128	256
		2 yr	32	512	64	256
		3 yr	32	256	64	256

[a] Immunoglobulin titers were determined with a fluorescein conjugate directed against both IgG and IgM. IgG and IgM titers were determined with class-specific antibody.

[b] Titer less than 16.

test (5, 7, 21), we attempted to determine whether ESS prepared from gradient-purified rickettsiae would sensitize sheep erythrocytes for agglutination by antibodies from several animal species. In one experiment rabbits were infected subcutaneously with *R. rickettsii* and bled from the heart 10, 20, 31, and 59 days later. The IHA antibody response of each rabbit is shown in Fig. 2. Antibodies were detected at 10 days in all four rabbits, reached a peak at about 20 days, and were declining slowly at 59 days.

Since the IHA test with our reagents successfully demonstrated *R. rickettsii* antibodies in all of the rabbit sera, we next determined the effect of substituting (i) glutaraldehyde-fixed sheep erythrocytes as used by Shirai et al. (21) for fresh erythrocytes and (ii) the Chang-type ESS used by Shirai for Anacker-type ESS. In the first experiment (Fig. 3) rabbit antibody titers obtained with Anacker-type ESS adsorbed to glutaraldehyde-fixed erythrocytes were generally similar to those obtained with Anacker-type ESS adsorbed to fresh cells. Glutaraldehyde-treated cells sensitized with Chang-type ESS were also agglutinated by high dilutions of the

TABLE 2. *Classes of human immunoglobulin which agglutinate human group O erythrocytes sensitized with antigen from R. rickettsii*

RMSF patient	Immunoglobulin[a]	IHA titer	Micro-IF titer[b]		
			IgG	IgM	IgA
G	IgG + IgA	256	512	32	0
	IgG	256	64	0	0
	IgM	1,536	24	192	0
H	IgG + IgA	4,096	2,048	64	0
	IgG	8,192	512	64	0
	IgM	≥49,152	0	768	0

[a] Immunoglobulins were separated on 10 to 40% sucrose density gradient and identified by immunodiffusion tests with heavy-chain-specific rabbit antibody.

[b] Titers were corrected for dilution of immunoglobulin during centrifugation in the gradient; 0 = titers of <32 for IgG + IgA and IgG fractions and <24 for IgM fraction.

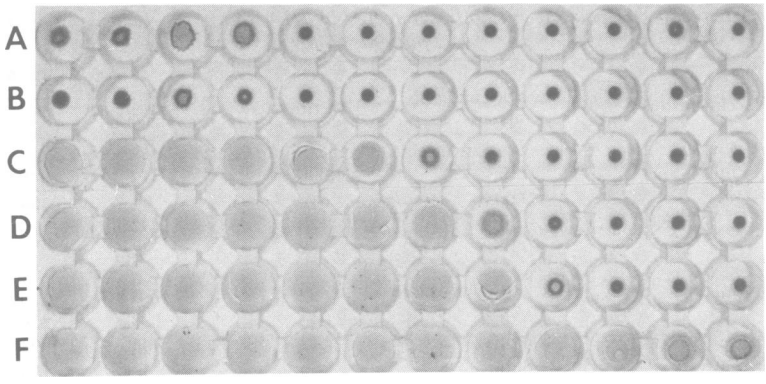

FIG. 1. *IHA test of immunoglobulin fractions of serum from RMSF patients 1 (rows A through C) and 2 (rows D through F) listed in Table 2. Rows: A and D, IgG and IgA; B and E, IgG; C and F, IgM. Fractions were diluted from 1:32 to 1:65,536 in rows A, B, D, and E and from 1:24 to 1:49,152 in rows C and F.*

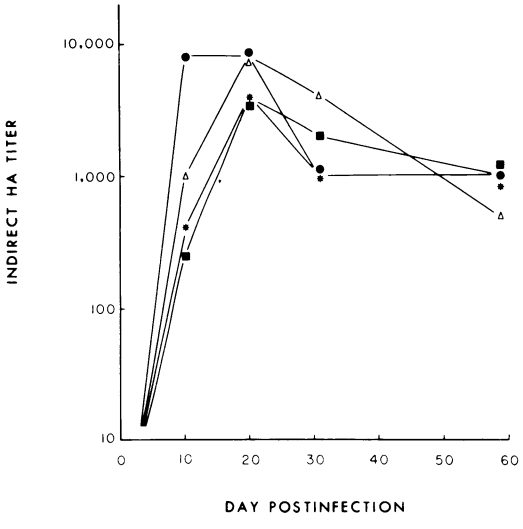

FIG. 2. *IHA titers of sera from individual rabbits inoculated subcutaneously with 1.1 × 10^3 plaque-forming units of R. rickettsii.*

rabbit sera (Fig. 4). Under the conditions of these experiments, the IHA test was uniformly successful in detecting rabbit *R. rickettsii* antibodies.

IHA antibody response of guinea pigs. Geometric mean serum titers for groups of five different guinea pigs bled at 7, 14, and 28 days after intraperitoneal infection with *R. rickettsii* are presented in Fig. 5. IHA antibody levels rose rapidly after day 7, peaked at about day 14, and declined rapidly. Results from one titration of the 14-day sera are shown in Fig. 6. CF antibodies were undetected at days 0 and 7, and peak levels did not occur until day 28 or later.

The suitability of glutaraldehyde-fixed erythrocytes and Chang-type ESS as reagents in the IHA test for titration of guinea pig antibodies was also determined. Fresh sheep erythrocytes sensitized with Chang-type ESS were agglutinated by guinea pig antibodies, but, in agreement with Shirai et al. (21), glutaraldehyde-fixed cells treated with either Anacker-type or Chang-type ESS were not agglutinated by immune guinea pig sera.

IHA antibody response of mice. Mice in groups of 15 were inoculated intraperitoneally with either a low dose (5.6 × 10^2 plaque-forming units) or a high dose (5.6 × 10^6 plaque-forming units) of *R. rickettsii*. Eleven days later, blood was obtained from the axillary plexus and pooled in groups of three. Little or no antibody was detected with the IHA test in sera from mice in the first group, but all five pools from the second group had IHA titers of 256 or higher (Fig. 7). A strong prozone was noted in every case.

DISCUSSION

IHA tests of sera from a limited number of human RMSF patients indicated a characteristic pattern of antibody response. In some cases antibodies were detected as soon as 3 to 6 days after onset of illness. They reached their highest levels in about 2 to 3 weeks and declined slowly over a period of many months. These results are in agreement with those of an earlier study with sera obtained up to 3 weeks after the first symptoms of disease (17). Micro-IF tests with specific conjugates for IgG and IgM revealed that the high IHA titers observed during the acute phase of illness correlated with the presence of IgM antibody. During convalescence, while micro-IF tests demonstrated relatively high levels of IgG antibodies in the serum, IHA titers dropped. Apparently, as has been shown in another system (11), IgM antibodies are more efficient than IgG antibodies in agglutinating erythrocytes.

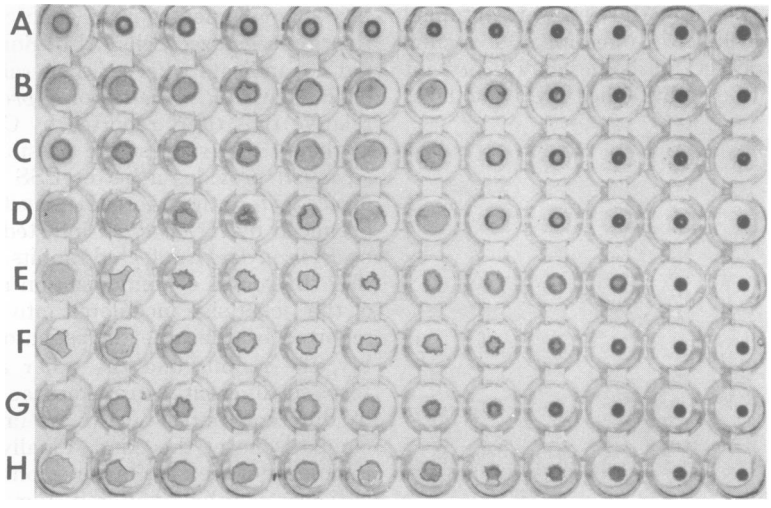

FIG. 3. *Effect of glutaraldehyde treatment of sheep erythrocytes on RMSF antibody titers of sera obtained from four rabbits 31 days after infection with 1.1 × 10^3 plaque-forming units of R. rickettsii. Individual sera (diluted 1:2 to 1:4,096) were tested against glutaraldehyde-treated sensitized erythrocytes in rows A through D and against unfixed sensitized erythrocytes in rows E through H.*

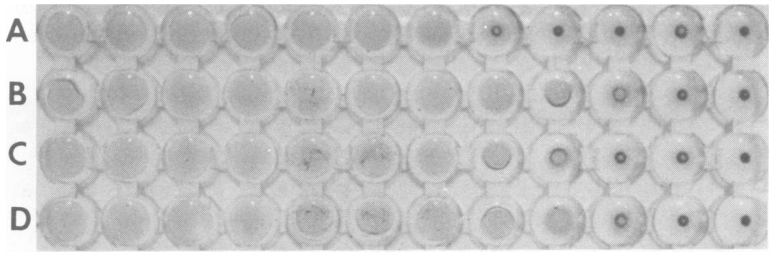

FIG. 4. *Agglutination of glutaraldehyde-fixed sheep erythrocytes sensitized with Chang-type ESS by sera obtained from four rabbits (rows A through D) 20 days after infection with 1.1 × 10^3 plaque-forming units of R. rickettsii. Individual sera were diluted 1:16 to 1:32,768.*

Whereas the highest IHA titers are associated with the presence of IgM antibodies, the highest micro-IF titers are associated with the presence of IgG antibodies. Since both IgG and IgM micro-IF titers depend essentially on a primary event, the simple binding of antibody to acetone-fixed rickettsiae, and IHA titers measure a secondary event, the agglutination of sensitized erythrocytes after prior binding of the IgM and IgG antibodies, which differ in their agglutinating efficiency, the micro-IF titers probably more accurately reflect the actual numbers of IgG and IgM antibody molecules in the sera. These differences in the antigen-antibody interactions in the two tests may in part be responsible for the fact that IHA titers are ofter higher than micro-IF titers during the earliest stages of RMSF and lower during the convalescent period, as shown here and in an earlier study (17).

In contrast to our success in demonstrating a strong antibody response to *R. rickettsii* by all of our infected rabbits with a variety of ESS-erythrocyte combinations, the positive results reported by others have been erratic. Chang et al. (7) found that one of two rabbits infected with epidemic typhus rickettsiae agglutinated specifically sensitized cells, even though both rabbits produced CF antibodies. However, in a later study with a more refined ESS preparation (5), they reported that all four rabbits inoculated with RMSF-group rickettsiae produced IHA antibodies. Shirai et al. (21) stated that only occasionally did their rabbit sera produce interpretable agglutination patterns.

Since the available experimental details of these other trials are limited, the reasons for the apparent discrepancies in results with rabbit antisera from the several laboratories are diffi-

cult to discern. Possibly, the sera were obtained a number of weeks after infection. Our results indicate that only IgG antibodies may be present at that time; these antibodies are capable of fixing complement but are inefficient at agglutinating sensitized cells. Also, Shirai et al. (21) did not indicate whether their rabbits were inoculated with *R. rickettsii* or *R. prowazekii*. The relative IHA titers reported by Chang and coworkers (5, 7) suggest the possibilities that *R. rickettsii* provides a better ESS than does *R. prowazekii* or that more IHA antibodies may be produced by *R. rickettsii*-infected rabbits than by *R. prowazekii*-infected rabbits, or both. Neither the routes of immunization nor the states of the rickettsiae inoculated into animals were reported by the other investigators. Conceivably, the rabbit (and the other animals) may respond differently to rickettsiae administered by different routes, but we are unaware of any comparisons of this kind. Finally, the genetic potentials of the rabbits to respond to rickettsial antigens may have differed from laboratory to laboratory. Various species differ in their abilities to produce antibodies useful for classification of rickettsiae; sera from mice are much better than those from guinea pigs for identifying species and types of RMSF-group rickettsiae by CF and micro-IF tests (16, 19).

Other workers have been even less sucessful

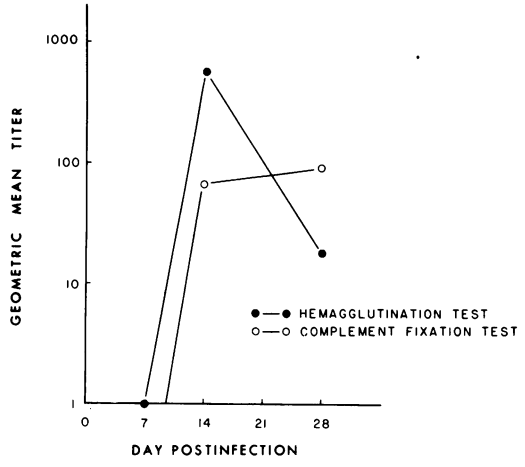

FIG. 5. *Serum antibody titers of guinea pigs infected intraperitoneally with 6.3 × 10^3 plaque-forming units of R. rickettsii. Each point represents the geometric mean titer of five guinea pigs bled only once.*

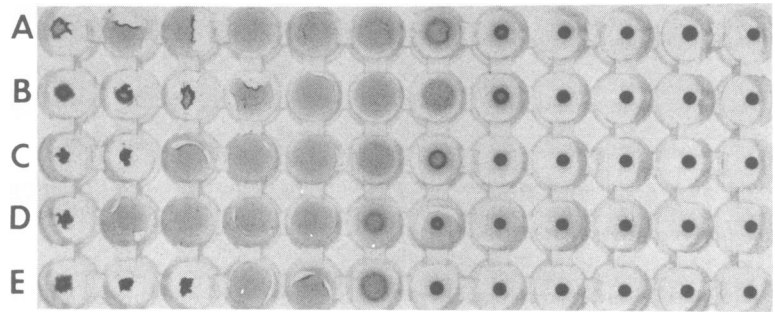

FIG. 6. *Agglutination of sensitized sheep erythrocytes by sera obtained from five guinea pigs (rows A through E) 14 days after intraperitoneal inoculation with 6.3 × 10^3 plaque-forming units of R. rickettsii. Individual sera were diluted 1:16 to 1:32,768.*

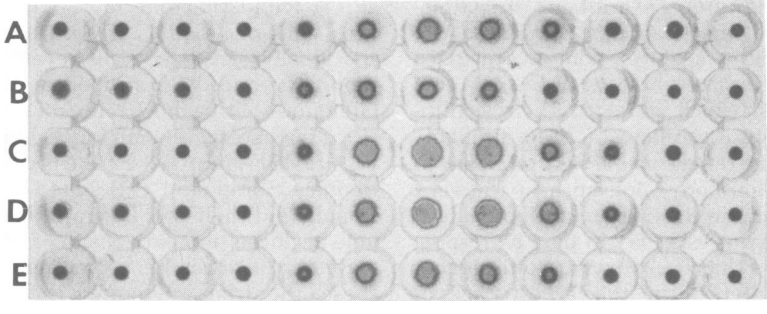

FIG. 7. *Agglutination of sensitized sheep erythrocytes by five pools (rows A through E) of sera obtained from mice 11 days after intraperitoneal inoculation with 5.6 × 10^6 plaque-forming units of R. rickettsii. Individual pools were diluted 1:2 to 1:4,096.*

at finding IHA antibodies in guinea pig sera than in rabbit sera. Chang et al. (5, 7) reported specific antibodies in only four of seven infected guinea pigs, and these titers were generally low (5 to 250). Also, only occasionally were Shirai et al. (21) able to find guinea pig antibodies capable of agglutinating sensitized glutaraldehyde-fixed erythrocytes. Our guinea pig sera also did not unequivocally agglutinate sensitized glutaraldehyde-fixed cells. However, when fresh erythrocytes rather than fixed erythrocytes were used, antibodies were found in one of five guinea pigs 7 days after infection and in five of five guinea pigs at both 14 and 28 days.

The reason why fixed cells are suitable for the IHA test with rabbit antisera but not with guinea pig antisera is not known. One may speculate that ESS contains at least two antigens, both capable of adsorbing to fresh sheep erythrocytes but only one capable of adsorbing to cells whose surfaces have been modified by glutaraldehyde. Furthermore, the rabbit produces antibodies to at least the antigen which adsorbs to both kinds of cells, but the guinea pig produces antibody to the antigen which adsorbs to fresh cells only. Some support for the concept of a minimum of two separate ESS is provided by a recent study by Osterman and Eisemann (15). They reported that radiolabeled ESS extracted from *R. prowazekii* and *R. conorii* were found in two distinct regions after centrifugation in a potassium tartrate density gradient. However, other interpretations of their data are possible, and additional work is necessary to determine the validity of the above hypothesis.

Mouse antibodies, at least antibodies from the random-bred RML mouse, are also amenable to study by the IHA test, although, compared with guinea pigs and rabbits, relatively large numbers of rickettsiae must be injected into mice to induce a response. Others have reported that mice are generally refractory to RMSF-group rickettsiae. Sammons et al. (20) found that only 2 of 35 mice, representing seven inbred strains, died after injection of 5×10^5 rickettsiae of the Sheila Smith strain of *R. rickettsii*. Also, to produce practical amounts of CF antibody to some members of the RMSF group, it is necessary to inject such large doses of rickettsiae that some of the mice die from rickettsial toxin rather than from an infectious process (13). Possibly, with limited multiplication of the rickettsiae in the resistant mouse, the low-dose inoculum simply does not provide a sufficient antigenic stimulus to trigger a detectable response.

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