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THE USE OF SOLID-PHASE RADIOIMMUNOASSAY TECHNIQUES FOR SERODIAGNOSIS OF HUMAN PLAGUE INFECTION

B. W. Hudson,[1] K. Wolff,[1] and T. Butler[2]

The results of solid-phase radioimmunoassay tests conducted with sera from confirmed and suspected human plague cases suggest such testing could have significant advantages for the diagnosis of this disease.

Introduction

Solid-phase radioimmunoassay (SPRIA) techniques have been valuable for quantifying antibody to bacteria (*1*) and viruses (*2,3*). In the indirect solid-phase radioimmunoassay technique, a purified antigen is usually attached directly to the solid phase. The attached antigen is then exposed to the human test serum; and after thorough washing, antibodies combined with the antigen are detected by means of radioisotopically labeled antibodies specific for human IgM or IgG.

Application of these techniques for determining the presence of human antibody to *Yersinia pestis* offers several apparent advantages. These are: the use of relatively moderate amounts of purified antigens; exquisite sensitivity—on the order of 0.5 μg of antibody per ml (*1*); and the ability to measure both IgM and IgG responses.

The soluble protein fraction 1A of *Y. pestis* (*4*), recommended for serodiagnosis of human plague infection (*5*), constitutes a highly purified specific antigen; but it is only available in limited quantities from noncommercial sources. The most sensitive serologic test now used for diagnosis of human plague, the passive hemagglutination (PHA) test (*5*), sometimes fails to detect serologic responses in human sera collected shortly after the onset of disease (*6,7*). Of particular concern is the PHA test's failure to detect titer increases in acute-to-convalescent sera from some vaccinated primates (*8*) or from occasional recurrent cases of human plague (*7*). Finally, it is difficult to adapt the PHA test to differentiation of human IgM and IgG responses, and a possibility exists that suitable measurement of these responses might have value in the serodiagnosis of human plague, particularly during early convalescence. For all of these reasons, we decided to use SPRIA techniques to examine a number of human sera available to us.

Materials and Methods

Sera

Most of the serum samples came from confirmed cases of human plague in Vietnam (*7*). These included acute and convalescent sera from 14 patients and convalescent sera from four other patients. An additional eight sera were obtained from North American patients with confirmed plague. These latter included an acute serum and two convalescent sera from one patient, and five convalescent sera from five other patients.

As negative controls we used the following sera, all collected in North America: three convalescent sera from patients with suspected plague infections later shown to be tularemia; seven convalescent sera from pa-

[1]Immunochemistry Branch, Vector-Borne Diseases Division, Bureau of Laboratories, Center for Disease Control, United States Public Health Service, Department of Health, Education, and Welfare, P. O. Box 2087, Fort Collins, Colorado 80522, U.S.A.

[2]Division of Geographic Medicine, Department of Medicine, Case Western Reserve University, Cleveland, Ohio 44106, U.S.A.

tients with suspected but unconfirmed plague; and three sera from family contacts of patients with suspected but unconfirmed plague.

Antigen

Purified protein fraction 1A of *Y. pestis* strain A 1122 was prepared using the methods of Baker (*4*). This preparation was homogenous when analyzed by sodium laurel sulfate acrylamide disc gel electrophoresis (*9*) and showed no cross-reactivity when used in the PHA test with hyperimmune *Yersinia pseudotuberculosis* or *Yersinia enterocolitica* sera (*5*).

Solid-phase Radioimmunoassay

The technique was adapted from that of Trent et al. (*10*). *Y. pestis* fraction IA (0.8 μg)—in 50 μl of phosphate-buffered saline (PBS) at pH 7.4, containing 0.1 per cent sodium azide, 2mM $MgCl_2$, and lmM $CaCl_2$—was placed in each well of 96-well microtiter plates treated for tissue culture (Linbro Scientific Co., Inc., Hamden, Conn.).[3] Plates containing antigen were air-dried overnight at room temperature. The next day 200 μl of 10 per cent normal rabbit serum in PBS (PBS-NRS) were added to each well and incubated 45 minutes at 37°C. The plates were then washed three times with PBS.

Sera to be tested were diluted 1:100 in PBS-NRS, and 50 μl of each specimen was added to each of four wells. For IgM determinations, the plates were then incubated for 5 hours at 37°C and 12 hours at 4°C, while for IgG subclass determinations they were incubated 1 hour at 37°C. All the plates were then washed three times with PBS.

Goat antisera specific for the heavy chains of human IgM and IgG were obtained from Antibodies, Inc., in Davis, California. These antisera had been purified on affinity columns by the manufacturer. After purification, the sera showed no cross-reactivity when tested by radial immunodiffusion, immunoelectrophoresis, and Ochterlon tests.[4]

The IgG-rich fractions of the antisera were prepared by ammonium sulfate precipitation (*11*), and protein concentrations were determined by a modified Folin method (*12*). These IgG fractions were then radioiodinated with ^{125}I (Amersham Searle) using the choramine T method (*3*). Antiglobulins were labeled with 1,000 microcuries of ^{125}I per 100 μg of protein in 1 ml volumes and were separated from free iodine on Sephadex G-25 columns. The resulting 5 to 6 ml amounts of iodinated globulin solution were diluted with an equal volume of normal rabbit serum (NRS). These stock solutions were then diluted in NRS for the SPRIA test (1:2 for IgM, 1:4 for IgG). Fifty μl of the ^{125}I-antiglobulin working dilution was added to each well of the microtiter plate involved. For IgM determinations the plates were incubated 5 hours at 37°C, and for IgG they were incubated 2 hours at 37°C. The plates then were washed three times with PBS and covered with plastic film; the wells were cut from the plate, placed in 20 ml scintillation vials, and counted in a Beckman Gamma-310 Counter. Means and standard deviations of the four determinations were used as a measure of ^{125}I antiglobulin binding for each serum.

Passive Hemagglutination (PHA)

In the PHA test, the purified fraction 1A and the standard methods recommended by the World Health Organization Expert Committee on Plague (*5*) were used.

Results

The SPRIA results obtained for the various categories of sera tested are summarized in

[3]Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health, Education, and Welfare.

[4]Detailed information about these tests can be obtained from the manufacturer.

Table 1. In tests with convalescent sera from patients with confirmed plague, the average binding of ^{125}I-labeled antihuman IgM and IgG reagents was two to six times higher than that obtained in tests performed with negative control sera from patients with suspected but unconfirmed plague. The range of values obtained overlaps, however.

In Table 1, assuming normal distribution of ^{125}I binding values, we have estimated the variance of each group of determinations and the 99 per cent confidence limits. The upper confidence limits for SPRIA binding of ^{125}I obtained using the negative control sera (IgM, 1,959 counts per minute [CPM]; IgG, 4,464 CPM) were found to lie below the lower confidence limits obtained using the convalescent sera from patients with confirmed plague (IgM, 2,790 CPM; IgG, 11,857 CPM). The upper confidence limits obtained in tests using the negative control sera therefore seem to provide reasonable criteria for categorizing sera as positive when tested by SPRIA.

We then classified all sera from patients with confirmed plague in terms of their positive or negative results when tested by SPRIA and PHA—considering IgM >1,959 CPM, IgG >4,464 CPM, and a PHA titer equaling or exceeding 1:16 as positive results. The outcome of this classification is shown in Table 2. PHA results were positive for eight of the 15 acute sera tested, while SPRIA results were positive for 10 of the 15. In tests with the convalescent sera, 23 of the 25 sera tested were positive by PHA, while all 25 were positive by SPRIA.

Since the sera were tested four times to obtain a measure of mean radioiodine uptake, we used Student's t test to compare values for acute and convalescent sera. Mean values differing at the 1 per cent significance level were classed as showing a rise in IgM or IgG levels. We analyzed data for the 15 cases in which both acute and convalescent sera were available (Table 3). Only one serum pair failed to show a significant increase in IgM or IgG levels. Both sera of this pair showed high levels of IgM and IgG, and a moderate but not statistically significant increase in both levels.

Four of the 15 serum pairs did not show fourfold increases in PHA titers. These sera are of particular interest in the comparative evaluation of SPRIA and PHA tests. Serum

Table 1. The results of IgM and IgG specific solid-phase radioimmunoassay (SPRIA) testing of sera from suspected and confirmed human plague cases.[a]

	SPRIA results, in counts per minute of ^{125}I		
	Results with 15 positive sera from acute confirmed plague cases	Results with 25 positive sera from convalescent confirmed plague cases	Results with 13 control sera from suspected but unconfined cases and family contacts
IgM test results, in counts per minute:			
Range	880-4,881	1,122-8,359	599-4,256
Mean	2,420	3,570	1,500
Standard deviation	181	302	178
99% confidence limits	1,953-2,886	2,790-4,349	1,041-1,959
IgG test results, in counts per minute:			
Range	2,094-29,274	2,468-35,849	993-5,303
Mean	6,585	19,574	2,895
Standard deviation	951	2,991	608
99% confidence limits	3,929-8,837	11,857-27,290	1,326-4,464

[a]The mean counts per minute and the pooled standard deviation for each group of sera were estimated on the basis of standard deviations from quadruplicate determinations for each serum. The 99% confidence limits were estimated using the pooled standard deviation and the standard normal distribution.

Table 2. A qualitative comparison of the test results obtained using solid-phase radioimmunoassay (SPRIA) and passive hemagglutination (PHA) on acute and convalescent sera from 25 confirmed cases of human plague.

	Passive hemagglutination (PHA) results			
	Acute sera (15 samples)		Convalescent sera (25 samples)	
SPRIA results	PHA-	PHA+	PHA-	PHA+
Negative (IgM −, IgG −)	4	1	0	0
Positive (IgM +, IgG −)	3	1	0	1
Positive (IgM −, IgG +)	0	2	2	4
Positive (IgM +, IgG +)	0	4	0	18
Total SPRIA positive	3	7	2	23

Table 3. Results of SPRIA testing of 15 paired acute and convalescent sera. In this table the paired sera have been classified as showing either significant increases or no significant increases in *Y. pestis* fraction 1A IgM or IgG antibody by Student's t test at the 1 per cent significance level.

		IgM, counts per minute			IgG, counts per minute		
Changes in IgM or IgG antibody levels	No. of serum pairs	Initial mean CPM	Final mean CPM	Mean CPM difference	Initial mean CPM	Final mean CPM	Mean CPM difference
No significant change in IgM or IgG	1	4,672	5,745	1,073	29,274	31,486	2,212
Significant IgM increase; no significant change in IgG	1	1,794	3,190	1,396	2,795	3,547	752
No significant change in IgM; significant IgG increase	4	3,251	3,064	-187	6,393	21,046	14,653
Significant IgM increase; significant IgG increase	9	1,869	4,355	2,486	4,234	17,273	13,039

from one other case is also of interest, although acute serum was no longer available. This serum came from a case in North America that was confirmed bacteriologically and was followed serologically for 3 months after onset. None of the sera collected demonstrated a significant PHA titer. One serum collected 30 days after onset yielded a PHA titer of 1:8.

Data on these five cases are shown in Table 4. All five yielded significant SPRIA results. Case 2 is particularly interesting, because the paired sera were from one of two cases of recurrent plague infection showing a *decrease* in PHA titer. Those two cases have been discussed elsewhere (7). These data suggest that, in comparison to PHA results, certain human convalescent sera may yield unusually high SPRIA results.

Figure 1 shows the PHA and SPRIA data for all the sera tested. In general, IgM and IgG levels in the sera seemed to be fairly well related to PHA titers. Seven of the 40 sera showed anomalously high IgM or IgG SPRIA values. The IgM and IgG values for these latter sera are plotted as open stars in the

Table 4. Passive hemagglutination (PHA) and SPRIA data from five confirmed cases of human plague. These cases were selected on the basis of low or reduced responses when originally tested by PHA.

Case No.	Type of serum	Days between date of onset and serum collection	PHA titer	SPIRA results[a] IgM[b] Counts per minute	IgM[b] Standard deviation	IgG[b] Counts per minute	IgG[b] Standard deviation
1	Acute	2	1:16	*3,301*	282	*5,538*	388
	Convalescent	17	1:32	*8,275*	564	*24,787*	5,720
	Convalescent	33	1:32	*6,778*	194	*26,599*	4,206
2	Acute	3	1:256	1,209	91	*8,109*	555
	Convalescent	8	1:64	1,122	126	*29,453*	3,573
3	Acute	2	1:16	*2,359*	149	3,872	549
	Convalescent	10	1:32	*2,972*	212	*5,114*	413
4	Acute	1	≤1:8	1,652	163	3,014	915
	Convalescent	14	1:16	1,225	209	*6,493*	1,784
5	Convalescent	30	1:8	756	44	*12,624*	685

[a]SPRIA results beyond the 99% confidence limit for unconfirmed cases are italicized (see text).
[b]CPM and the standard deviation for each serum were estimated on the basis of standard deviations from quadruplicate determinations.

figure. All seven were convalescent sera—four being from three of the five cases presented in Table 4. Of these seven sera, SPRIA-IgG data from six seemed to have a distribution different than data from the remaining sera. The regression line for these six appears as the dashed line in Figure 1B. If the data from these seven sera are deleted, analysis of data from the 33 remaining acute and convalescent sera shows a correlation between the observed PHA titers and the IgM and IgG levels (the correlation coefficients being 0.658 for IgM and 0.894 for IgG). SPRIA IgG and PHA data are more closely correlated than SPRIA IgM and PHA.

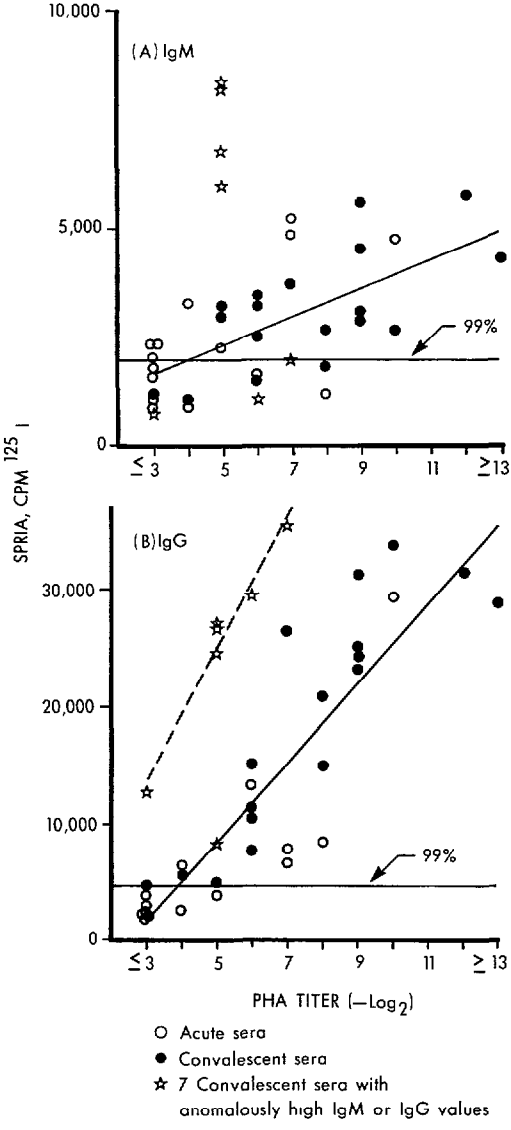
Analysis of IgG and PHA data for the seven anomalous sera gives a correlation coefficient of 0.745. If the one serum showing high IgM levels but "normal" IgG levels is excluded from this sample, data from the remaining six sera yield a correlation coefficient of 0.984.

Discussion

Difficulties in using the PHA test for serodiagnosis of plague infection have frequently been pointed out. Thus, Marshall and coworkers (*6*) emphasized that 35 of 69 Vietnamese patients, from each of whom two sera were obtained, showed no significant rise in titer. In addition, 59 of 71 single-serum specimens from patients with bacteriologically proven plague were seronegative. The serum specimens used in these studies were collected during early convalescence, and the authors indicate that during early convalescence the serum antibody response may be delayed. This delay may be complicated by actual decreases in titer in populations with preexisting titers. Two cases of recurrent plague infection studied by Butler (*7*) in Vietnam showed actual drops in PHA titers. Similar results have been reported in vaccinated primates when challenged with living plague bacteria (*8*). Obviously, this type of result, although infrequent, could cause diagnostic difficulties, particularly in vaccinated individuals.

Some of these difficulties could perhaps be avoided by use of SPRIA techniques. Serum samples from one patient with recurrent plague showed highly significant increases in IgG levels when tested by SPRIA, and four additional cases—samples from which were negative by PHA or showed no significant in-

Figure 1. A scatter diagram comparing data obtained by PHA and SPRIA analysis of acute and convalescent sera from confirmed plague cases. (A) SPRIA-determined IgM levels compared to PHA titers; (B) SPRIA-determined IgG levels compared to PHA titers. The two horizontal lines represent the upper 99 percent confidence limits of the SPRIA data obtained from sera of unconfirmed suspected plague cases and family contacts (see text and Table 1). The two solid diagonal lines show the least squares linear regression fits for 33 sera. The dashed line in Figure 1B is the regression line for six convalescent sera with unusually high SPRIA binding values (see text and Table 4).



creases in PHA titer—were confirmable by SPRIA tests (Table 4).

A second consideration relates to data obtained by analyzing 13 sera from unconfirmed suspect plague cases. Two convalescent sera in this group of 13 gave SPRIA results above the estimated upper 99 per cent confidence limit for the group. One serum was obtained from a patient suspected on good epidemiologic evidence of having contracted plague from rabbits in New Mexico. Serologic tests (PHA) and bacteriologic tests were negative for plague, but a moderate titer of 1:128 to tularemia was found. This serum yielded IgM SPRIA levels of 4,256 CPM and negative IgG results. Bacteriologic and serologic results for the second suspect case, a patient in California, were negative. This serum gave negative IgM SPRIA results but moderately positive SPRIA IgG levels of 5,303 CPM. Naturally, this initial investigation does not provide grounds for accurately defining the criteria for use of SPRIA in diagnosing human plague; but it does suggest that the first-mentioned unconfirmed case may have been misdiagnosed.

The possibility that serologic diagnosis of human plague could be improved by suitable application of SPRIA techniques is of obvious importance to those engaged in control of this disease. In addition, conserving scarce antigen and serum samples is important for laboratories engaged in plague diagnosis. A total of 6.4 μgm of fraction 1 antigen and 4 μl of serum were needed to test the IgM and IgG content of each serum specimen. Although equipment costs may limit the use of SPRIA tests to better-equipped reference laboratories, the potential for conserving antigen and serum specimens may prove an advantage to such laboratories.

SUMMARY

Solid-phase radioimmunoassay (SPRIA) techniques were used to analyze the levels of IgM and IgG antibody to purified *Yersinia pestis* fraction 1A in various human sera. The results were compared with data obtained by using the standard passive hemagglutination (PHA) test recommended by the World Health Organization Expert Committee on Plague. These results suggest that use of SPRIA might help conserve the commercially unobtainable fraction 1A antigen. In addition, it appears that certain human plague cases that fail to show significant PHA titers or fail to exhibit increases in PHA titers can be diagnosed by suitable use of SPRIA.

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