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Radioimmunoassay for Quantitation of Antibodies to Alphaviruses with Staphylococcal Protein A

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A radioimmunoassay (RIA) procedure is described for measuring antibodies to alphaviruses in human and other mammalian sera. The test employed protein A-bearing *Staphylococcus aureus* as a solid-phase immunoadsorbent for ^{3}H-labeled viruses complexed with immunoglobulin G. Using antibodies produced in humans and guinea pigs, the RIA procedure clearly differentiated among antibodies to Venezuelan, western, and eastern equine encephalomyelitis viruses. Sensitivity of the RIA depended on the concentrations of labeled viruses employed. The dilution of serum that effected binding of 50% of the ^{3}H-labeled virus (determined by probit analysis) was consistently higher than the neutralizing antibody titer determined by a conventional plaque reduction neutralization test using 80% plaque reduction end points. In addition, sera from 73 individuals were screened for seroconversion following live attenuated Venezuelan equine encephalomyelitis virus vaccine (strain TC-83) inoculation, by RIA using a single serum dilution (1:80); results were identical with seroconversions identified by plaque reduction neutralization test. Hyperimmune Venezuelan equine encephalomyelitis virus sera from a number of mammalian species were successfully titrated by RIA; the species tested were human, guinea pig, white rat, rabbit, burro, dog, monkey, sheep, and cotton rat. The protein A-mediated RIA is a rapid, sensitive, specific, and precise serological tool for measuring antibodies to surface antigens of alphaviruses, and should allow the subsequent development of a competitive binding RIA to measure antigenic potency of inactivated alphavirus vaccines.

Protein A is a molecule present in high concentration on the surface of certain *Staphylococcus aureus* strains (16). It rapidly binds most mammalian immunoglobulin G (IgG) molecules (14) through an interaction with the Fc region. Formalin-fixed staphylococci retain protein A on their surfaces (11), and may be used as solid-phase adsorbents in radioimmunoassay (RIA) procedures to separate virus-antibody complexes from unbound radio-labeled virus by low-speed centrifugation. Protein A-bearing *Staphylococcus* thus substitutes for the secondary antibody (anti-IgG) in an RIA, and offers several advantages. Since protein A combines with most mammalian IgG molecules, it is not necessary to prepare individual precipitating anti-IgG antibodies for each species tested. The reaction between IgG and protein A is very rapid (3); 1- to 10-min incubation times are sufficient, compared to the 12 to 18 h required for the secondary antibody technique. The protein A *Staphylococcus* reagent is also easily and economically prepared (3).

This paper reports the development of RIA procedures using protein A-bearing *Staphylococcus* to measure antibodies against alphaviruses in human and other mammalian sera. These procedures were adapted from published methods using protein A to measure antibodies to α-fetoprotein (10), bovine serum albumin, bacterial and tumor-associated antigens (1), and mycoplasma (2). In our hands, the test is rapid, sensitive, specific, and precise, and should be a useful tool for measuring the antibody responses of humans and other mammals to alphavirus infections.

MATERIALS AND METHODS

Radiolabeled virus preparations and assays. Venezuelan equine encephalomyelitis virus (VEE) strain Trinidad donkey, western equine encephalomyelitis virus (WEE) strain 72V1880, and eastern equine encephalomyelitis virus (EEE) strain NJ 1945 were used at the passage levels stated previously (5, 6). To prepare ^{3}H-labeled viruses, all viruses were passaged once in BHK-21 cells grown in 800-cm^2 roller bottles. Following virus adsorption, the culture medium was replaced with medium 199 containing Earle salts and 1:40-concentration amino acids. At 4 to 5 h after inoculation, ^{3}H-labeled L-amino acids were added to each roller bottle (500 μCi per bottle), and incubation was continued for 24 h. Cell culture supernatant fluids were harvested, concentrated, and purified by

rate zonal centrifugation as previously described (8). Infectious virus was assayed by counting plaque-forming units (PFU) in primary duck embryo cell culture monolayers maintained in 10-cm^2 wells of plastic plates under medium containing 1% agarose (7). Radioactivity was measured by counting 50- or 100-μl samples dissolved in 6 ml of Scintolute and Scintosol (4:1) in a Searle Mark II scintillation counter.

Preparation of *S. aureus* immunoadsorbent (SaCI). *S. aureus* Cowan I (ATCC 12598) was originally obtained from the American Type Culture Collection and was grown in medium which consisted of 17 g of Trypticase, 10 g of yeast extract, 5 g of NaCl, and 2.5 g of K_2HPO_4 per liter of distilled water. Cultures were lyophilized and maintained as described previously (9). Bacteria from reconstituted ampoules were grown in Erlenmeyer flasks on a shaker at 37°C for 24 h and harvested by an adaptation of the method published previously (3). Briefly, the bacteria were centrifuged at 4,000 × *g* for 10 min, washed in Hanks balanced salts solution (HBSS) and resuspended in ⅛ the original volume in HBSS containing 1.5% formaldehyde. The suspension was stirred for 90 min at room temperature, washed, resuspended in HBSS, heated to 80°C for 3 min, cooled in an ice bath, and recentrifuged. The wet weight of the final bacterial pellet was determined, and the bacteria were resuspended in HBSS to 10% wt/vol. This suspension was divided into small volumes and frozen and stored at −60°C until ampoules were thawed immediately prior to use. In the text, this reagent will be referred to as SaCI.

Titration of immune sera using SaCI. Twofold serial dilutions of sera were prepared in RIA buffer, which consisted of HBSS containing 0.5% bovine serum albumin and 10 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.6. To each 0.45-ml volume of serum dilution, 50 μl of ^{3}H-labeled virus (diluted in RIA buffer to an appropriate concentration, usually 8.1 $\log_{10}$ PFU/ml, as described in the text) was added. Labeled virus and serum were incubated for 1 h at 37°C. Following incubation, 100 μl of each mixture was removed for assay and replaced with 100 μl of SaCI. Each tube was briefly mixed using a Vortex shaker and centrifuged at 2,500 rpm for 10 min. A 100-μl sample of each supernatant fluid was then assayed for residual (unbound) ^{3}H-virus activity; the proportion of virus bound to SaCI was calculated. Under the conditions stated, less than 5% of the labeled VEE and WEE was bound nonspecifically. However, for ^{3}H-EEE, greater proportions often bound nonspecifically. This nonspecific adsorption could be inhibited by the addition of 0.2 M potassium phosphate buffer (pH 7.6) to the RIA buffer. In block titrations where EEE was included, this 0.2 M phosphate buffer was substituted for the standard buffer, with no effect on the VEE and WEE RIA end points.

An RIA titer was defined as the dilution of serum expected to effect 50% binding of ^{3}H-labeled virus to SaCI. This value was obtained by probit analysis of all data points for each curve. Although probit analyses were routinely performed by computer, close approximations could also be obtained by plotting data points on probit paper.

Immune sera. Hyperimmune antisera were produced in a number of experimental animals (listed in Tables 1 and 2) by subcutaneous inoculation of formaldehyde-inactivated alphaviruses, followed by subcutaneous challenge with live, virulent strains of VEE (Trinidad donkey), WEE (B-11), or EEE (Cambridge). The hyperimmune human serum was obtained from a laboratory worker who was immunized with the live, attenuated VEE vaccine, strain TC-83, and who subsequently acquired a laboratory infection with an undetermined VEE virus strain. Other human sera were obtained from Institute personnel who had been inoculated with VEE vaccine, or with inactivated WEE or EEE vaccines, as part of the routine immunization procedure for "at risk" laboratory workers. All sera were routinely heated (56°C, 30 min) prior to testing by RIA or plaque reduction neutralizing antibody (PRN) tests, except in one experiment where heated and freshly obtained sera were compared. The PRN tests were performed using sera and challenge viruses diluted in HBSS with 1% bovine serum albumin (pH 7.6). Challenge viruses were VEE strain TC-83, WEE strain 72V1880, or EEE strain NJ1945. Between 75 and 150 PFU of each virus per 0.2 ml were incubated with equal volumes of serum dilutions for 1 h at 37°C. Following incubation, virus-serum mixtures were assayed for residual PFU on primary duck embryo cell culture monolayers. The highest dilution of serum that reduced the challenge PFU by 80% or more was considered the PRN end point.

Chromatographically purified human IgG was obtained from Miles Laboratories, Inc. The IgG concentration in this preparation and in normal human serum was determined by radial immunodiffusion using a kit supplied by Miles Laboratories, Inc.

RESULTS

Application of SaCI precipitation techniques to measurement of alphavirus antibodies in hyperimmune guinea pig sera. Figure 1 illustrates the use of SaCI to precipitate VEE virus-antibody complexes and thereby to measure VEE antibody in hyperimmune guinea pig serum. The proportion of radiolabeled virus bound was a function of antiserum dilution. Figure 1 represents an average of six replicate determinations for each serum dilution. (The results are pooled from three titrations using duplicate serum dilutions, run on different days by the same technician.) The dilution of serum that effected 50% binding was determined by probit analysis to be 1:44,565. The 95% confidence interval for this point ranged from 1:38,862 to 1:50,268 (i.e., less than one twofold dilution). Thus the RIA titer for this serum was 1:44,565 against ^{3}H-VEE antigen.

The RIA titer, however, was a function not only of the specific antibody concentration but also of the antigen concentration employed. As shown in Fig. 2, when lower concentrations of VEE antigen were employed, less antibody was required to bind 50% of the labeled antigen, and higher RIA end points were obtained. This relationship between RIA titer and antigen con-

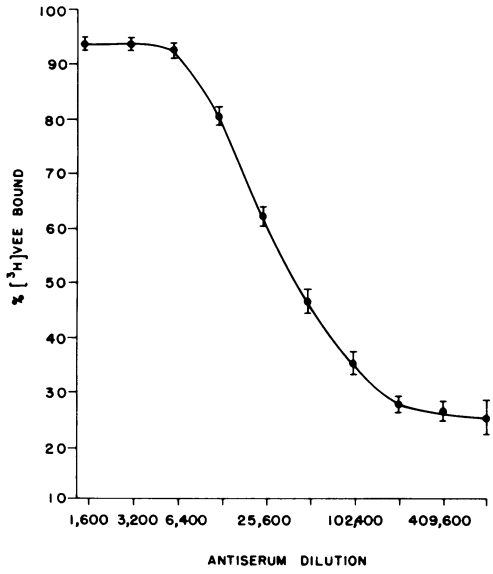

FIG. 1. *Precipitation of ^{3}H-VEE-antibody complexes from hyperimmune guinea pig serum dilutions using 1× SaCI and 8.1 $\log_{10}$ PFU of ^{3}H-VEE.*

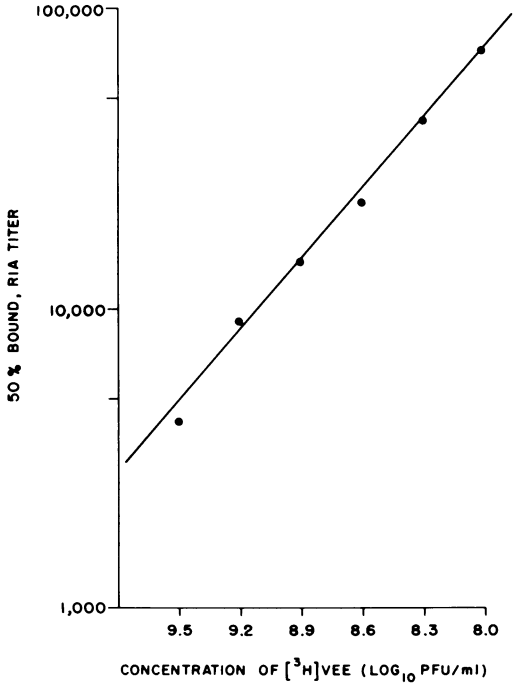

FIG. 2. *RIA titers of hyperimmune guinea pig serum, as a function of ^{3}H-VEE concentrations employed in the SaCI precipitation procedure.*

centration must be considered when the specificity of a given antiserum is titrated against a series of closely related antigens. Thus, it was a necessary prerequisite to adjust the antigens tested to a uniform concentration using independent criteria such as PFU, total virus particles, or total virus protein.

In Table 1, the specificities of three hyperimmune guinea pig sera were tested by RIA against homologous and heterologous alphaviruses. The three ^{3}H-alphavirus preparations were adjusted to a concentration of 8.1 $\log_{10}$ PFU/ml. This concentration was selected because it represented the maximum dilution of labeled antigen that contained approximately 10,000 cpm of ^{3}H. In a block titration, the combination of viruses with their homologous antisera clearly resulted in greater binding than did heterologous combinations. In fact, little to no cross-reactivity was detected. The test therefore appeared to be useful for differentiating among antibodies against VEE, WEE, and EEE. The effects of varying certain conditions of the procedure were next investigated. The titration illustrated in Fig. 1 was repeated with the incubation conditions changed from 37°C for 1 h, as in Fig. 1, to 5°C for 18 h. The RIA titer based on three replicate determinations for this serum under the latter conditions was 46,892, versus 44,565 in Fig. 1, an insignificant difference ($P < 0.05$). For convenience, we routinely employed the conditions of 37°C for 1 h.

Comparative RIA titrations were also performed using heated and unheated immune sera. A portion of freshly obtained immune guinea pig serum was heated (56°C, 30 min) while the

TABLE 1. *Separations of virus-antibody complexes from mixtures of ^{3}H-labeled alphaviruses and homologous or heterologous hyperimmune guinea pig sera*

Antiserum	Dilution	% ^{3}H-labeled virus bound[a]		
		VEE	WEE	EEE
VEE	100	85.9	7.0	11.2
	1,000	82.6	2.4	5.1
	10,000	49.1	4.4	2.1
WEE	100	6.3	87.0	9.2
	1,000	6.3	77.2	2.0
	10,000	5.3	39.7	0.1
EEE	100	2.1	0.4	92.5
	1,000	1.4	0.1	52.8
	10,000	0.9	0.3	11.8
Nonimmune	100	3.6	5.9	5.7
	1,000	1.4	4.9	3.7
	10,000	1.7	4.8	3.9

[a] ^{3}H-labeled virus preparations were adjusted to contain 8.1 $\log_{10}$ PFU/ml. The RIA diluent in this experiment contained 0.2 M potassium phosphate buffer.

remainder was stored at 5°C. Both portions were then titrated in triplicate, and RIA end points were compared. In this test, the heated portion titered 1,894 (standard deviation = 306) and the unheated portion titered 1,870 (standard deviation = 351), indicating that the presence of complement had no effect.

One additional variable was also examined. Formaldehyde-inactivated ^{3}H-VEE antigen (0.05% formaldehyde, 37°C, 18 h) was substituted for live virus in repeating the guinea pig serum titration in Fig. 1. Again, no significant difference was obtained for the end points determined using live or formaldehyde-inactivated virus antigens.

Application to other mammalian sera. The applicability of the protein A-mediated RIA methodology to several other mammalian species was tested using hyperimmune anti-VEE sera. Table 2 compares the titers with conventional PRN titers for these same sera. Control sera (not listed), obtained from nonimmunized animals of each species, were uniformly negative by both tests. The RIA titers exceeded PRN titers in every case. The RIA/PRN ratios are listed to illustrate this point; however, since only one hyperimmune serum was tested for each species, the ratios listed characterize only the sera tested and should not be construed as representative for all immune sera of that species.

Application to human sera. In Fig. 3, the complete titration curves are presented for two sera. Serum no. 2 is the same hyperimmune human serum listed in Table 2. Serum no. 1 was obtained from a human 14 days after inoculation with VEE vaccine, strain TC-83. Probit analyses, performed using the portion of each curve where the slope was negative, indicated RIA titers of 35 and 1,017 for sera no. 1 and 2, respectively. However, for both sera, less ^{3}H-VEE was bound by high serum concentrations than by intermediate serum dilutions.

The data in Table 3 are from an experiment

TABLE 2. *Comparative sensitivities of the PRN and protein A-mediated RIA tests for measuring VEE antibodies in selected mammalian sera*[a]

Species	Titer		RIA/PRN ratio
	RIA	PRN	
Human	1,071	160	6.69
Rhesus monkey	11,384	1,024	11.11
Guinea pig	44,565	5,120	8.70
Rabbit	18,749	2,048	9.15
White rat	11,481	1,280	8.96
Cotton rat	1,155	160	7.21
Dog	3,698	320	11.55
Sheep	1,840	320	5.75
Burro	814	160	5.05
Horse	420	80	5.25

[a] ^{3}H-VEE, 8.1 $\log_{10}$ PFU/ml.

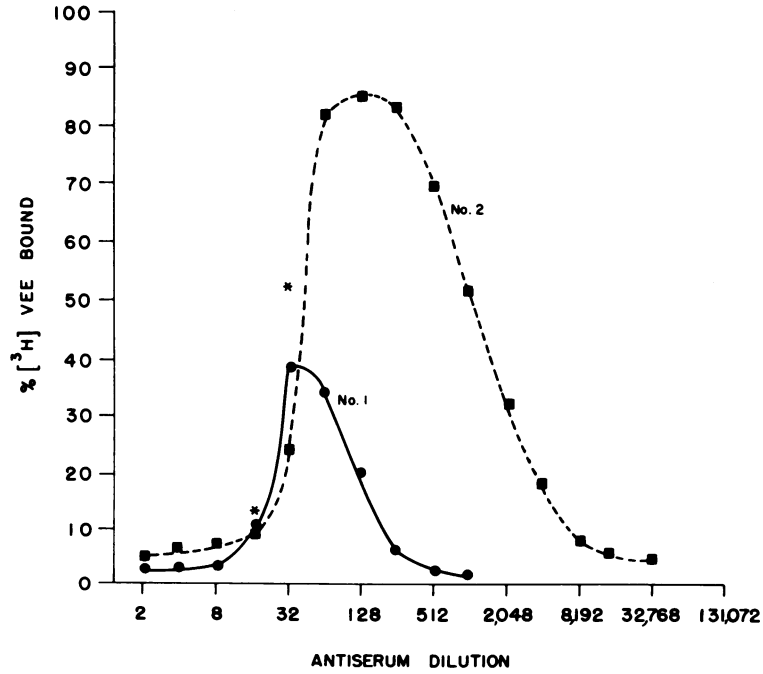

FIG. 3. *Complete RIA titers of two human sera, using 8.1 $\log_{10}$ of ^{3}H-VEE and 1× SaCI. *, percentage bound by serum no. 1 when 2× SaCI was substituted for 1× SaCI.*

TABLE 3. *Inhibition of binding ^{3}H-VEE-antibody[a] complexes to S. aureus immunoadsorbent by IgG or nonimmune serum*

Inhibitor	IgG concn (mg/ml)[b]	Corresponding serum dilution	% ^{3}H-VEE antibody bound	
			SaCI 1×	SaCI 2×
Nonimmune serum	1.71	1:8	0.9	1.5
	0.85	1:16	1.5	6.9
	0.43	1:32	34.4	76.3
	0.21	1:64	62.7	84.6
	0.10	1:128	79.5	79.5
	0.05	1:256	75.4	78.9
IgG	2.00		0.8	1.1
	1.00		2.0	7.6
	0.50		2.1	15.8
	0.25		51.1	81.0
	0.12		76.2	84.4
	0.06		78.4	NT[c]
	0.03		77.5	NT
None			78.6	80.1

[a] ^{3}H-VEE-antibody complexes were formed by incubating (37°C, 1 h) a 1:5,000 dilution of serum no. 2 (Fig. 3) with 8.1 $\log_{10}$ PFU of ^{3}H-VEE.
[b] Determined by radial immunodiffusion.
[c] NT, Not tested.

designed to explain the zone of inhibition observed for high serum concentrations. In this experiment, labeled VEE was combined with a 1:500 dilution of serum no. 2 in the presence of various concentrations of purified IgG or nonimmune serum. Following incubation, SaCI was added in the usual (1×) concentration (10% wt/vol) or in a 2× concentration (20% wt/vol). A significant inhibition of binding of ^{3}H-VEE-antibody to 1× SaCI was observed when IgG concentrations exceeded 0.25 mg/ml, or when whole serum concentration exceeded 3%. This inhibition was partially reversed by the addition of higher SaCI concentrations. Thus, using 1× SaCI, a significant inhibition of binding can be expected in serum dilutions lower than 1:64. As denoted by * in Fig. 3, increased binding was observed when 2× SaCI was used to test a 1:32 dilution, but not lower dilutions, of immune sera. In titrations of human sera, we routinely tested twofold dilutions beginning at 1:64 or 1:80, using 1× SaCI. If lower dilutions (1:32 or 1:40) were tested, the 2× SaCi was substituted.

Table 4 compares the results of the RIA and PRN tests for sera from 17 individuals obtained as early as 14 days to as late as 11 years following VEE strain TC-83 vaccine inoculation. Using 8.1 $\log_{10}$ PFU/ml of radiolabeled VEE as the antigen, RIA curves were generated, and 50% bound end points were determined by probit analysis. For all 17 sera, RIA end points exceeded PRN titers. On the average, RIA end points were 3.2 times higher (range, 1.32 to 6.01). Preimmunization sera from all 17 individuals were negative by both tests.

The protein A-mediated RIA was also a useful procedure for demonstrating seroconversions of 73 individuals inoculated with VEE strain TC-83 vaccine. Pre- and post-inoculation sera were tested by RIA at a single dilution, 1:80, and results were compared with conventional PRN test results. We regarded binding of greater than 50% as positive and less than 20% as negative. Since in our experience nonimmune sera do not bind greater than 20%, in this screening procedure sera that bound greater than 20% but less than 50% were scored as equivocal. All 73 preinoculation sera were scored negative by both tests. Of the 73 post-inoculation sera, 63 were scored positive by both tests (Table 5). Eight individuals failed to seroconvert by either test. Two sera were negative by the PRN test and equivocal by RIA. No sera were scored positive by PRN and negative by RIA; likewise, no sera were scored positive by RIA and negative by PRN. Thus, there was a very close association between seroconversions detected by the PRN and RIA procedures.

Finally, the specificity of the RIA screening procedure was tested for a limited number of human sera. Sera from four individuals inocu-

TABLE 4. *Comparative titers of VEE antibodies in human sera using the PRN and protein A-mediated RIA tests*[a]

Serum no.	Titer		Ratio of RIA/PRN
	RIA	PRN	
1	1,025	640	1.60
2	486	160	3.03
3	754	160	4.71
4	290	160	1.81
5	180	40	4.50
6	848	640	1.32
7	1,317	640	2.05
8	7,695	1,280	6.01
9	732	320	2.28
10	612	320	1.91
11	733	160	4.58
12	210	40	5.25
13	2,611	640	4.07
14	281	160	1.75
15	8,098	5,120	1.58
16	110	20	5.50
17	93	20	4.65
Average			3.32

[a] Sera were obtained from laboratory personnel 14 days to 11 years following inoculation of VEE vaccine, strain TC-83.

TABLE 5. *Comparisons of PRN and RIA tests for detecting seroconversions[a] among individuals inoculated with VEE vaccine strain TC-83*

Results of tests		No. of post-inoculation sera in category
PRN[b]	RIA[c]	
+	+	63
−	−	8
−	±	2
+	−	0
−	+	0

[a] All 73 preinoculation sera were scored negative by both PRN and RIA tests. All sera were tested at 1:80 dilution only.

[b] +, ≥80% plaque reduction in serum dilution ≥1:10.

[c] +, ≥50% ^{3}H-VEE bound; −, <20% bound; ±, ≥20% bound <50%.

lated with WEE and EEE, but not VEE, vaccines and screened by RIA were scored positive against WEE and EEE antigens but negative against VEE. Conversely, sera from 32 individuals inoculated with only VEE vaccine were positive against VEE, but negative against WEE and EEE antigens.

DISCUSSION

In these studies, an RIA procedure is described for measuring specific antiviral antibodies against VEE, WEE, and EEE viruses in guinea pig and human sera. The test depends on the use of protein A-containing *S. aureus* as a solid-phase immunoadsorbent. The assay offers several advantages over the conventional PRN test. The chief advantage is that the entire test can be performed and results obtained in less than 2 h. Results are very reproducible; titers obtained in replicate tests rarely vary by as much as one twofold dilution. The test is economical; the SaCI reagent is easily prepared (3), and radiolabeled virus can be diluted 50- to 100-fold, costing less than $0.01 per tube for ^{3}H. Most importantly, the test is sensitive and specific. The test clearly differentiated antibodies to three alphaviruses (VEE, EEE, and WEE) in guinea pig and human sera; titers obtained using the RIA techniques were consistently higher than PRN test end points. Sensitivity could presumably be increased even further if radiolabeled viruses with higher specific activity were prepared; such preparations could be diluted further and would require less antibody to effect 50% binding (Fig. 2). The selection of radioisotopes and labeling procedures is beyond the scope of this paper. However, if the reagents are to be used for routine serology, consideration should be given to the half-life of the radioisotope employed. For example, we have obtained results equal to or better than those obtained with ^{3}H-labeled viruses by substituting ^{32}P-labeled viruses with extremely high specific radioactivities. However, the short half-life of ^{32}P (13.6 days) precludes the use of ^{32}P-labeled viruses for routine serological applications.

The close correlation between the results of the PRN and RIA tests, when used to measure the serological responses of individuals inoculated with VEE vaccine, documents the validity of the RIA procedure. Whereas the RIA is more sensitive than the PRN test for detecting antibody in high dilutions of serum, the presence of an inhibitor, probably excess immunoglobulin, in low dilutions of serum restricts the usefulness of the RIA for detecting antibody in serum dilutions of 1:32 or less. Acetone or kaolin extraction procedures, routinely used to remove nonspecific inhibitors from sera prior to testing by the hemagglutination-inhibition test, did not reduce the degree of inhibition noted in the RIA test. This limitation in testing low dilutions of sera may be offset in part by the addition of more protein A. In hypergammaglobulinemic sera, the zone of inhibition would presumably be greater.

The specificities of protein A for various immunoglobulins are not yet completely defined. For human and guinea pig sera, protein A reacts principally with IgG_1, IgG_2, and IgG_4 (4, 12); however, binding with certain human IgM and IgA myeloma proteins has been reported (14). Thus the "early" antibody detected 14 days after vaccine inoculation could conceivably be IgM or an appropriate subclass of IgG.

The protein A of *Staphylococcus* has been reported to bind to the Fc region of immunoglobulins from most mammalian species (13). Only serum from the American opossum (*Didelphis marsupialis*) fails to react, although sera of other species, including sheep, goat, and deer, react poorly. It has been reported that sera from rats, horses, sheep, cows, and chickens do not compete with human IgG for binding sites on *S. aureus* (15), but this observation may only reflect lower avidities rather than absolute nonreactivity of these species with protein A. For example, we have successfully titrated sheep and rat sera for VEE antibodies using protein A. Other investigators have reported weak but detectable reactions of bovine and sheep sera with protein A (15); these reactions apparently depended on IgG_2 but not IgG_1 in those species. Although for some species only minor proportions of the total immunoglobulin may react with protein A, this may be sufficient for detecting antibody to alphaviruses. Immune sera from each animal species will have to be tested individually. However, it is reasonable to predict

that sera from most natural mammalian reservoir hosts of alphaviruses will react to some extent with protein A, thus rendering the RIA methodology useful for epidemiological investigations of alphavirus ecology.

In conclusion, the protein A-mediated RIA is a rapid, sensitive, and specific serological tool for measuring antibodies to alphaviruses in a variety of mammalian sera. One further distinct advantages of the RIA procedure over the PRN test for alphaviruses antibodies is that Formalin-inactivated antigens may be substituted for infectious virus, thus reducing the risk of laboratory-acquired infections. Preliminary data not reported here suggest that this procedure may be sufficiently specific to differentiate among antibodies to the serologically distinct enzootic and epizootic strains of VEE, which would further extend its usefulness in epidemiological investigations.

One further extension of the procedures described will be the development of competitive binding assays to measure minute quantities of viral antigens in candidate inactivated alphavirus vaccine preparations. Such an assay, if successfully developed, might supplement or eventually replace the mouse potency assay for assessing potential vaccine efficacy.

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