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Factors Involved in Enzyme-Linked Immunoassay of Viruses and Evaluation of the Method for Identification of Enteroviruses

JOHN E. HERRMANN,* ROGER M. HENDRY, AND MARILYN F. COLLINS
Department of Microbiology, Harvard School of Public Health, Boston, Massachusetts 02115

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A quantitative enzyme-linked immunosorbent assay was used for identification of selected enteroviruses: poliovirus type 1, echovirus type 6, coxsackievirus A type 9, and coxsackievirus B types 1 through 6. Partially purified viral antigens or virus-specific antibodies were adsorbed to polystyrene spectrophotometer cuvettes, which permitted the assays to be reported and compared in terms of enzyme units specifically reacting. Both the adsorbed antigen and the adsorbed antibody methods were approximately equal in terms of sensitivity and specificity of reaction. By use of [^{14}C]leucine-labeled enteroviruses, the amount of virus that bound to the plastics used was shown to be dependent on the purity of the virus preparation used, but it was higher than the amount that was bound by plastics coated with viral antibody. Diluents which contained 0.15% (vol/vol) Tween 20 and 2.0% (wt/vol) bovine serum albumin in phosphate-buffered saline, pH 7.2, were found to be the most effective in inhibiting nonspecific adsorption of immunoreagents. However, the presence of these inhibitors in phosphate-buffered saline solutions also caused desorption of virus or viral antibody during immunoassays; the amount of virus desorption varied with the type of preparation used, and antibody desorption was dependent on the concentration of antibody initially used for adsorption. For specific identification of a given enterovirus type by the enzyme-linked immunosorbent assay method used, approximately 10^5 plaque-forming units of virus per assay tube were required.

Solid-phase enzyme immunoassays, usually called enzyme-linked immunosorbent assays (3) or, more recently, enzyme-linked immunospecific assays (1), have been applied to the detection of antibodies to a variety of microbial and parasitic infections, as well as to toxin detection and assays of hormones and a number of other chemical substances. The application of the method to virus identification is more limited, but it has been used to identify plant viruses (21), hepatitis B antigen (22), hepatitis A antigen (12), herpesviruses (14), and human reovirus-like agent (23).

For enterovirus identification, the most common method used is virus neutralization, which, because of the large number of enterovirus types, can be both time-consuming and costly. Immunofluorescent (4, 16, 20) and immunoperoxidase techniques (9) for enterovirus identification have been described, but they require subjective judgments, which are always a factor in histochemical tests. For this reason, the suitability of the enzyme-linked immunoassay for use in enterovirus identification was investigated. The present paper describes the identification of selected enterovirus types by this technique and the factors that are involved in the assay.

MATERIALS AND METHODS

Viruses and tissue cultures. Poliovirus types 1 and 2, echovirus type 6, coxsackievirus B types 1 and 4 through 6, and coxsackievirus A type 9 were obtained from the National Institutes of Health (NIH) Research Resources Branch, Bethesda, Md. Coxsackievirus types B2 and B3 were obtained from D. O. Cliver, University of Wisconsin, Madison. Viruses were propagated in Vero cells (obtained from D. O. Cliver) or in BGM cells (obtained from R. S. Safferman, Environmental Protection Agency, Cincinnati, Ohio).

Virus antigens. Virus was inoculated onto cell monolayers in 75-cm^2 flasks and incubated with serum-free minimal essential medium, 5 ml per flask. After the cells showed 3 to 4+ cytopathic effects, they were frozen and thawed twice. Cellular debris was removed by centrifugation at 40,000 × *g* (Sorvall RC-2B centrifuge) for 30 min. Five grams of anion exchanger (Bio-Rad AG2-X8) was washed in distilled water and added to 30 ml of virus solution. The mixture was stirred with an overhead stirrer at 300 rpm for 1 h and filtered through a Millipore fritted-glass filter. By plaque titration, no virus losses were noted by use of the anion exchange resin or the filtration step. Uninfected cells

were processed in the same way for use as cell antigen controls.

Virus antigens were also prepared by extraction with diethyl ether. Virus suspensions clarified by centrifugation as above were mixed with an equal volume of cold (4°C) ether and held on ice for 2 h. The aqueous layer was collected, and the residual ether was removed under vacuum.

Radioactive virus. For preparation of [^{14}C]leucine-labeled poliovirus 2 and coxsackievirus B3, BGM or Vero cultures were starved of leucine for 18 h by incubation with Earle balanced salt solution. The cultures were inoculated with virus suspended in Earle balanced salt solution at a concentration of ca. 10 plaque-forming units (PFU) per cell, adsorbed for 30 min at room temperature, and rinsed twice with Earle balanced salt solution. [*U*-^{14}C]leucine (specific activity, 270 mCi/mmol; New England Nuclear Corp., Boston, Mass.) in Earle balanced salt solution (20 μCi/ml) was added with or without 1 μg of actinomycin D per ml, and the cultures were incubated for 24 h at 37°C. The cultures were frozen and thawed three times, and the harvested virus was centrifuged at 2,500 × *g* to remove cell debris. The supernatant fluids were collected, and the labeled virus was purified (5) by passage through a diethylaminoethyl-Sephadex A-50 column (1.5 by 30 cm), using 0.06 M phosphate buffer, pH 7.5, as the eluent. The effluent column fractions in which peak counts per minute (Searle model 6880 liquid scintillation counter) and peak virus PFU coincided were the fractions used as labeled virus.

Sera. Viral antisera used were horse antiviral sera obtained from the NIH Research Resources Branch or rabbit antiviral sera obtained from Microbiological Associates (Bethesda, Md.). For use in the assays, viral antisera were absorbed with cell debris before use. This was done by freezing and thawing cell cultures, centrifuging the cell debris at 2,500 × *g*, and suspending the pellet (0.1 ml) in 1 ml of antisera diluted 1:10 with phosphate-buffered saline, pH 7.2 (PBS), plus 1% bovine serum albumin (BSA). The cell-sera mixture was incubated for 24 h at 4°C, and the cell debris was removed by centrifugation at 2,500 × *g*. Sera were tested for absence of antibody to cell antigens by enzyme immunoassay before use.

Enzyme-labeled antibodies. Globulin fractions of rabbit anti-horse immunoglobulin G (IgG) and goat anti-rabbit IgG (Antibodies, Inc., Davis, Calif.) were obtained by $(NH_4)_2SO_4$ precipitation (40% saturation) of whole sera. The method for coupling the globulins with enzyme (horseradish peroxidase, code HPOFF; Worthington Biochemicals Corp., Freehold, N.J.) was by use of periodate (15). Peroxidase-labeled globulin was separated from unlabeled material by gel filtration on columns (2.5 by 80 cm) of Sepharose 6B. Fractions that gave maximum absorption in a spectrophotometer at both 403 nm (enzyme) and 280 nm (protein) were pooled and precipitated by addition of $(NH_4)_2SO_4$ to 40% saturation. The precipitate was suspended in a volume of PBS to give a protein concentration of approximately 2 mg/ml and dialyzed against PBS for 3 days at 4°C. The preparations were tested for immunological reactivity in gel diffusion plates against the appropriate globulin and stored at −20°C until used.

Enzyme immunoassay. The procedure for solid-phase enzyme-linked immunoassay was based on that of Engvall and Perlmann (3), as modified by Ruitenberg et al. (17). Virus antigens diluted 1:4 in PBS were added to wells of polystyrene microtiter plates (Cooke Engineering, Alexandria, Va.), 0.1 ml per well, or to polystyrene spectrophotometer cuvettes (Variable Volumetrics, Inc., Woburn, Mass.), 0.2 ml per cuvette. Both plates and cuvettes were pretreated with 25 μg of poly-L-lysine per ml in PBS (10) to enhance antigen binding. The antigens were adsorbed for 1 h at 37°C plus overnight at 4°C.

The plates or cuvettes were washed three times with distilled water or PBS plus 0.05% (vol/vol) Tween 20. Dilutions of test and control sera were added (0.05 ml per well, 0.1 ml per cuvette) and incubated for 30 min at 37°C. The samples were washed as above, and peroxidase-conjugated antiglobulin was added (0.05 ml per well, 0.1 ml per cuvette) at a 1:100 dilution. The diluent for the sera and the conjugates was PBS with 2% (wt/vol) BSA and 0.15% (vol/vol) Tween 20 added. Optimal dilutions of viral antigens and immunoreagents used were determined by "checkerboard" titrations. For the microtiter plate assay, the plates were incubated for 30 min at 37°C and washed as above, and 0.2 ml of substrate (0.05% 5-aminosalicylic acid in distilled water adjusted to pH 6.0 with 1 N NaOH plus 0.005% H_2O_2) per well was added plus 1 drop of 1% (wt/vol) gelatin to help prevent precipitation of the reaction product. After 30 to 60 min at room temperature, the reaction was stopped with 1 drop of 1.5 M sodium azide. A red-brown reaction product was formed; the endpoints were read visually by comparison with controls (antigen plus normal serum or heterotypic viral antisera or both and antigen plus PBS).

For spectrophotometric assays, the cuvettes were incubated at 37°C for 30 min and washed as above, 0.1 ml of phosphate buffer (0.01 M, pH 6) was added, and 2.9 ml of enzyme substrate (3×10^{-4} M *O*-dianisidine dihydrochloride, 0.001 M H_2O_2) in the same buffer was added. The reaction was monitored (absorbance at 460 nm) with a Zeiss PM6-KS recording spectrophotometer, and enzyme units were calculated. One unit of peroxidase was the amount of enzyme decomposing 1 μmol of H_2O_2 per min at 25°C under the conditions above.

For comparison with the above method, an adsorbed antibody method was also used. Virus antisera (horse) at a 1:50 dilution were adsorbed to cuvettes as above. After adsorption and washing of the samples, ether-extracted virus preparations diluted 1:4 in PBS plus 2% BSA and 0.15% Tween 20 were added (0.1 ml per cuvette), and the mixtures were incubated for 1.5 h at room temperature. The samples were washed with PBS-Tween, 0.1 ml of diluted rabbit antiviral sera (1:100) was added, and the mixtures were incubated for 30 min at 37°C. Subsequent treatments and addition of conjugates and enzyme substrates were the same as those for the adsorbed antigen method above. In addition to the use of poly-L-lysine to enhance antibody binding to plastics, glutaraldehyde was also tested as described previously (18).

RESULTS

Virus purification procedures. Because there is a limited amount of protein that can be

adsorbed to plastics (7), viral antigens were partially purified. The efficiency of the virus purification procedures for removing cellular protein was tested with uninfected BGM cell cultures treated in the same manner as virus-infected ones. The amount of protein removed after each treatment with Bio-Rad AG 2X8 anion exchange resin or by ether extraction was measured by absorption at 280 nm and the method of Lowry et al. (11). The protein standard used for comparison was BSA. One extraction with ether removed as much protein as did one ion exchange treatment (Table 1). Subsequent ether extractions (not shown) did not give additional protein reduction, but further ion exchange treatments did. The same extraction procedures used for virus preparations did not cause any loss of virus as measured by the plaque method. For use in enzyme immunoassay tests below, one ether extraction or two anion exchange treatments proved to be sufficient.

Nonspecific adsorption. To test for nonspecific adsorption of immunoreagents to plastics, peroxidase-labeled anti-rabbit globulin was diluted in PBS with various amounts of BSA and Tween 20 added and adsorbed to spectrophotometer cuvettes (1 h at 37°C). The substrate addition and reading of results were as described for enzyme immunoassay tests above.

At lower concentrations of BSA and Tween 20, some peroxidase-labeled antiglobulin adsorbed to polystyrene spectrophotometer cuvettes nonspecifically (Table 2). Tween 20 at 0.15% (vol/vol) plus BSA at 2% (wt/vol) gave about as low a background as did any combination tried. Also, these concentrations did not interfere with antigen-antibody reactions, as measured by precipitin tests and enzyme immunoassay tests. Thus, we selected this combination as a standard diluent (PBS-BSAT diluent) for both viral antibody and peroxidase-labeled antiglobulin.

Antibody adsorption. To determine the effect of antibody concentration on adsorption to polystyrene and to find if the above diluent would cause elution of antibody adsorbed to plastics, rabbit anti-horse IgG labeled with peroxidase was added to normal rabbit IgG to give final IgG concentrations of 2, 10, and 100 μg/ml. These were adsorbed to polystyrene tubes in the same manner as that which was used for an immunoassay (1 h at 37°C plus overnight at 4°C). The tubes were incubated for 1 h at 37°C with diluents (PBS with BSA or Tween 20 or both) and washed, and enzyme substrate was added. The product was determined spectrophotometrically, and the number of enzyme units bound to the tubes was calculated. The results are shown in Table 3. It can be seen from the table that the diluent used did not cause elution of adsorbed antibody at 10 μg/ml, but did cause some elution at the higher level (100 μg/ml). This could cause some loss of sensitivity if the serum antibody to be adsorbed needed to be used at a low dilution (≤1:100), assuming an IgG level of 10 mg/ml in serum. Prior treatment of the tubes with BSA and glutaraldehyde (18) did not increase the number of enzyme units that could be adsorbed.

TABLE 1. *Virus antigen purification procedure: removal of cellular protein*

Procedure	Protein	
	Concn (mg/ml)	Reduction (%)
Centrifugation[a]	0.61	
Ion exchange, extraction no.:		
1	0.42	31
2	0.26	57
3	0.15	75
Ether extraction	0.38	38

[a] Cells were removed from suspension by centrifugation at 40,000 × *g* for 30 min.

TABLE 2. *Inhibition of nonspecific adsorption of immunoreagents*

Inhibitors[a]		Enzyme conjugate adsorbed (enzyme units)
BSA (%, wt/vol)	Tween 20 (%, vol/vol)	
0	0	0.50
1	0	0.16
2	0	0.08
3	0	0.11
4	0	0.06
0	0	0.58
0	0.05	0.28
0	0.10	0.04
0	0.15	0.02
0	0.20	0.02
2	0	0.08
2	0.05	0.03
2	0.10	0.02
2	0.15	0.01
2	0.20	0.01

[a] Inhibitors were added to PBS in the amounts indicated.

Adsorption of enteroviruses. The sensitivity of a solid-phase immunoassay depends in part on the amount of antigen adsorbed to the solid-phase surface or to an antibody-coated surface. The amount of enterovirus that adsorbed was determined by use of [^{14}C]leucine-labeled coxsackievirus type B3 or [^{14}C]leucine-labeled poliovirus type 2 or both. For direct antigen adsorption, labeled virus at a 1:4 dilution in PBS was adsorbed to polystyrene tubes, as described

TABLE 3. *Adsorption of peroxidase-labeled antibody to polystyrene tubes*

PBS diluent added		Enzyme units bound (%)[a] at antibody protein concn of:		
BSA (%)	Tween 20 (%)	2 μg/ml	10 μg/ml	100 μg/ml
0	0	42.6	44.4	47.2
0	0.15	38.5	42.8	27.1
1	0	38.5	50.1	37.3
1	0.15	38.1	47.6	30.1
2	0	30.9	49.0	33.1
2	0.15	31.7	41.5	29.0

[a] Based on the number of units bound divided by number of units in adsorbing solutions after treating tubes coated with peroxidase-labeled antibody for 1 h at 37°C with the diluent indicated.

in Materials and Methods for enzyme immunoassay, and either tested for virus adsorbed directly or incubated with PBS-BSAT diluent before assay. The bottoms of the tubes were cut off, dissolved in scintillation fluid, and counted. Both types of labeled viruses, in which >95% of the radioactivity is virus associated (6) when purified by column chromatography, adsorbed better when used alone than when partially purified (batch method ion exchange resin) unlabeled virus was added (Table 4). This might be due to the relatively high amounts (ca. 250 μg/ml) of cellular protein present in unlabeled preparations. For both types of viruses and both types of preparations used, there was considerable elution of adsorbed virus by incubation with PBS-BSAT diluent. Based on the initial infectivity titer (PFU per milliliter) of input virus used, the maximum amount remaining adsorbed in unlabeled preparations would be 1.0×10^6 PFU per tube for poliovirus type 2 and 8.1×10^5 PFU per tube for coxsackievirus B3. Virus also adsorbed to the glass tubes used as controls. There was less initial adsorption for most samples, but virus which did adsorb was less readily eluted.

For comparison with the direct adsorption of virus to plastics above, the uptake of virus by antibody-coated tubes was measured. Labeled coxsackievirus B3 and labeled plus unlabeled coxsackievirus B3 were diluted as above in PBS-BSAT diluent, incubated for 1.5 h at room temperature, and washed, and the tubes were assayed for bound counts per minute. The maximum amounts of virus uptake were 5.2% of input virus for labeled virus alone and 4.1% for labeled plus unlabeled virus (Table 5). Thus, the amount of virus available for immunoassay is approximately the same as that obtained by direct adsorption above for unlabeled plus labeled virus but is less for the labeled virus alone. The data also show that, because there was only a slight increase in uptake of the labeled virus alone, purity of virus, i.e., absence of cell protein, is not as important a factor in this type of assay, which is based on an immune reaction for virus binding, as it is in others which are not. Glutaraldehyde (data not shown), used either directly or in conjunction with adsorbed BSA, did not increase the uptake of labeled virus by either of the two methods used.

Virus identification. The microtiter plate method was used as a preliminary test to ensure that viral antisera did not visibly react with

TABLE 4. *Adsorption of [^{14}C]leucine-labeled enteroviruses to polystyrene tubes*

Virus prepn[a]	Virus adsorbed (%)[b]					
	Polystyrene		Treated polystyrene[c]		Glass	
	I[d]	E[e]	I	E	I	E
^{14}C-labeled PO-2	65.5	30.8	64.5	40.85	29.1	28.9
^{14}C-labeled PO-2 + PO-2[f]	11.8	5.5	11.6	6.1	18.3	14.3
^{14}C-labeled CB-3	65.9	34.2	68.6	38.0	32.7	23.9
^{14}C-labeled CB-3 + CB-3[f]	20.1	6.3	24.1	8.1	17.0	15.4

[a] PO-2, Poliovirus type 2; CB-3, coxsackievirus B type 3.

[b] (Counts per minute of adsorbed virus/counts per minute of input virus) × 100.

[c] Tubes treated with poly-L-lysine as described in the text.

[d] I (initial), percentage of input counts per minute adsorbed to tubes after three washes with PBS plus 0.05% Tween 20.

[e] E (eluted), percentage of input counts per minute adsorbed to tubes after incubation for 1 h at 37°C with PBS-BSAT and washing as in footnote *d*.

[f] [^{14}C]Leucine-labeled enteroviruses plus unlabeled enterovirus at the concentration used for immunoassays.

TABLE 5. *Binding of [^{14}C]leucine-labeled coxsackievirus type B3 (CB-3) by antibody-coated tubes*

Virus prepn	Anti-CB-3 dilution adsorbed	cpm bound (%)[a]
^{14}C-labeled CB-3	1:10	1.4
	1:50	5.2
	1:100	1.5
	None	0.2
^{14}C-labeled CB-3 + CB-3[b]	1:10	1.2
	1:50	4.1
	1:100	0.9
	None	0.5

[a] (Counts per minute bound to tubes/counts per minute of input virus) × 100.

[b] [^{14}C]Leucine-labeled CB-3 plus unlabeled CB-3 at the concentration used for immunoassay.

cellular antigens, to demonstrate reactivity of viral antisera with specific virus types, and to determine the optimal dilutions of viral antigens and immunoreagents. The spectrophotometric assay, which yields quantitative data, was used for the virus identifications reported here. Enzyme units bound were calculated for both positive (type-specific) sera and control sera.

To determine the positive-negative (P/N) ratio required for these assays to be considered positive, an enzyme-linked immunoassay for coxsackievirus type B2 was used. Viral antigen was adsorbed to polystyrene cuvettes in replicate samples. Sera used were rabbit anti-coxsackievirus B2 and normal rabbit serum, both diluted 1:100 in PBS-BSAT. The indicator of the reaction was peroxidase-labeled goat anti-rabbit IgG. The data obtained are shown in Table 6. From these data, it was calculated by the Mann-Whitney test that the two groups of values represented distinct populations at the 95% confidence level and that the difference between a test and control sera would need to be 0.07 enzyme units for a positive identification. Thus, from the value shown for normal rabbit serum, the P/N ratio for a test serum would need to be 2.0 or higher to be considered positive.

For identification of selected enterovirus types (poliovirus type 1, coxsackievirus types B1, B2, and A9), the adsorbed antigen method was used.

TABLE 6. *Precision of enzyme-linked immunoassay for coxsackievirus type B2*

Serum	Replicate	Enzyme units bound (mean ± standard deviation)
Normal rabbit serum	1	0.073
	2	0.065 (0.071 ± 0.006)
	3	0.076
Anti-coxsackievirus B2	1	0.625
	2	0.552 (0.584 ± 0.037)
	3	0.575

Homotypic and heterotypic rabbit antiviral sera were used; the indicator of the reaction was peroxidase-labeled goat anti-rabbit IgG, as above. All of the viruses tested were positively identified when compared with normal serum, giving P/N ratios of 2.5 or higher, and all gave higher P/N ratios with homotypic than with heterotypic sera (Table 7). Some of the heterotypic sera did give stronger reactions than did normal serum, but, with the exception of the two coxsackie B viruses, all had P/N ratios of less than 1.7. The higher values obtained with some of the heterotypic sera could indicate a degree of antigenic relatedness, but more extensive studies with viral antigens of greater purity would be required before any definitive conclusions are reached. For the coxsackie B viruses, there was significant cross-reaction between types B1 and B2. Subsequent testing of all the viruses in the B group (data not shown) indicated that these viruses could be accurately identified as to group only. Reaction of B-group viral antigens with antisera to virus types in other groups was minimal, as measured by either microtiter plate assays (not shown) or by spectrophotometric tests (Table 7).

To compare the adsorbed antigen method with the adsorbed antibody method, enzyme-linked immunoassays were tested on several virus types. Both methods gave approximately the same results for identification of echovirus type 6 poliovirus type 1, coxsackievirus B1, and coxsackievirus A9, with P/N ratios of >2.0 (Table 8). Cell antigen preparations were tested against virus-specific antisera for all types and gave P/N ratios varying from <1.0 to the 1.3 value shown for anti-coxsackievirus A9 in Table 8.

Sensitivity of assays. The sensitivity of the assays for enteroviruses, in terms of the number of PFU of virus required to give a positive test, was determined for coxsackievirus A9. Viruses were adsorbed to polystyrene tubes at dilutions

TABLE 7. *Reaction of enterovirus types[a] with type-specific and heterotypic antisera by enzyme-linked immunoassay*

Viral antiserum used	Virus antigen adsorbed[a]							
	PO-1		CB-1		CB-2		CA-9	
	EU[b]	P/N[c]	EU	P/N	EU	P/N	EU	P/N
PO-1	0.32	2.9	0.17	1.4	0.23	1.6	0.09	1.3
CB-1	0.09	0.8	0.30	2.5	0.21	1.5	0.08	1.1
CB-2	0.17	1.5	0.25	2.0	0.47	3.4	0.10	1.5
CA-9	0.14	1.3	0.10	0.8	0.19	1.4	0.24	3.4
Normal rabbit serum	0.11		0.12		0.14		0.07	

[a] PO-1, poliovirus type 1; CB-1, CB-2, and CA-9, coxsackievirus types B1, B2, and A9.
[b] EU, Enzyme units bound.
[c] P/N, EU bound with viral antiserum (positive)/EU bound with normal rabbit serum (negative).

TABLE 8. *Comparison of adsorbed antigen and adsorbed antibody methods for enterovirus identification by enzyme-linked immunoassay*

Antigen tested[a]	Method					
	Adsorbed antigen			Adsorbed antibody		
	Enzyme units bound			Enzyme units bound		
	Type-specific antiserum	Normal rabbit serum	P/N[b]	Type-specific antiserum	Normal rabbit serum	P/N
EC-6	0.28	0.08	3.5	0.27	0.12	2.3
PO-1	0.34	0.11	3.1	0.24	0.11	2.2
CB-1	0.31	0.13	2.4	0.19	0.09	2.1
CA-9	0.50	0.12	4.2	0.43	0.08	5.3
Cell extract[c]	0.15	0.11	1.3	0.10	0.08	1.3

[a] EC-6, Echovirus type 6; PO-1, poliovirus type 1; CB-1, coxsackievirus type B1; CA-9, coxsackievirus type A9.

[b] Enzyme units bound with viral antiserum (positive)/units bound with normal rabbit serum (negative).

[c] Serum used for cell extract was anti-CA-9.

of 1:5 to 1:500 from initial concentrations of 2.0 $\times 10^7$ PFU/ml for coxsackievirus A9 and 5.6 $\times 10^7$ PFU/ml for echovirus 6, which was used as a virus control. Cell extract was also tested at the same dilutions, as a control for the possible presence of cell antibody in viral antisera. Rabbit anti-coxsackievirus A9 serum was added at a 1:100 dilution, followed by goat anti-rabbit IgG labeled with peroxidase. The substrate used to indicate presence of bound enzyme was 0.08% (wt/vol) 5-aminosalicylic acid in distilled water at pH 6.0 plus 0.005% H_2O_2. The absorbance at 460 nm was measured after 15 min of reaction. The highest dilution of coxsackievirus A9 giving absorbance values at 460 nm 2.0 or more times that of either the echovirus 6 or the cell antigen control was between 1:200 and 1:100 (Fig. 1), which is equivalent to 1.0×10^5 to 2.0×10^5 PFU per assay tube.

A similar assay was done to determine the titer of antiserum used for coxsackievirus A9 identification. Virus or cell antigen was adsorbed to polystyrene tubes at a 1:10 dilution, and rabbit anti-coxsackievirus A9 serum was added at dilutions from 1:50 to 1:1,600. The titer of the antiserum used, after absorption with cell debris, was 1:1,600 as determined by plaque reduction. Serum controls tested were rabbit anti-coxsackievirus B1 serum and normal rabbit serum. Peroxidase-labeled antiglobulin and 5-aminosalicylic acid substrate were added as above. Up to a 1:800 dilution of anti-coxsackievirus A9 serum was clearly positive (P/N = 2.1 from data), when compared with the control that was most strongly reactive (Fig. 2).

DISCUSSION

The enzyme-linked immunoassay technique used was found suitable for type-specific identification of the enterovirus types selected and for group identification of coxsackie B viruses. The reason for the lack of specificity within the B group is not clear, but it may be related to the viral antigens that adsorb most strongly to the plastics or to the antisera used or to both. Group antigens, which could adsorb more strongly, have been demonstrated for these viruses (19), and for diagnosis of coxsackie B viruses by immunofluorescence neither horse nor rabbit antisera were found entirely satisfactory for type-specific identification, whereas hamster antisera and mouse immune ascitic fluids were (4).

Because of the low amount of viral antigen present in enterovirus preparations (ca. 1 ng/10^8 virions) and because some of the types are antigenically related, immunoassays of these viruses require optimal conditions. It was found that, for direct adsorption of viral antigen to polystyrene tubes or plates, some type of partial purification was necessary. Extraction of the virus samples with ether or a batch method

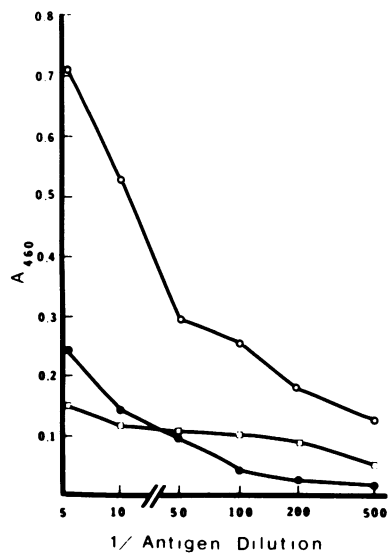

FIG. 1. *Detection limits of coxsackievirus type A9 by enzyme-linked immunoassay. Virus concentration per sample tube at a 1:5 dilution was 4 $\times 10^6$ PFU. Symbols: ○, coxsackievirus A9 antigen reacted with anti-coxsackievirus A9 serum; ●, coxsackievirus A9 antigen reacted with anti-echovirus type 6 serum; □, cell antigen reacted with anti-coxsackievirus A9 serum. A_{460}, Absorbance at 460 nm.*

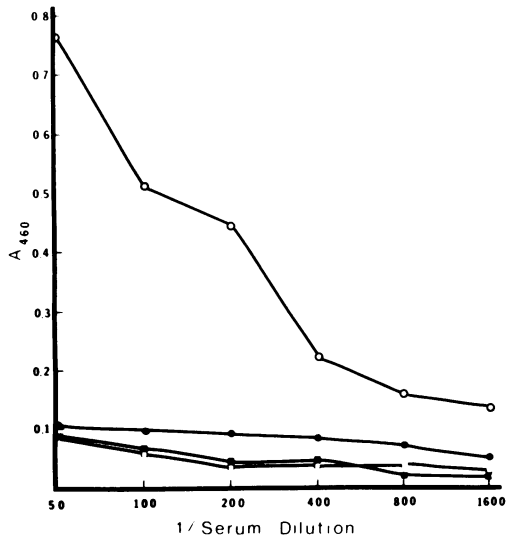

FIG. 2. *Titration of antibody to coxsackievirus type A9 by enzyme-linked immunoassay. Coxsackievirus type A9 was used at a 1:10 dilution, as was cell antigen. Symbols: ○, coxsackievirus A9 antigen reacted with anti-coxsackievirus A9 serum; ●, cell antigen reacted with anti-coxsackievirus A9 serum; □, coxsackievirus A9 antigen reacted with anti-coxsackievirus type B2 serum; ■, coxsackievirus A9 antigen reacted with normal serum. A_{460}, Absorbance at 460 nm.*

treatment with an anion exchange resin removed sufficient cellular protein to permit adsorption, although maximum adsorption was obtained with virus purified by passage through a column of diethylaminoethyl-Sephadex A-50. For routine diagnostic work the simpler methods are sufficient, and no gain in accuracy was obtained by use of purified virus (data not given).

The precision of the assay was high, when a given set of reagents were tested under the same conditions. Over a period of time, when different reagents are used, the precision would undoubtedly be less, although our background values for normal sera have been quite similar over periods of several months. Most reports consider a P/N ratio of 1.8 or more to indicate a positive result in an immunoassay. The ratio selected, though, depends on the precision of an individual set of assays, as there is not as yet a standard method. The precision can be increased by decreasing nonspecific adsorption; we found that use of PBS-BSAT diluent for diluting immunoreagents gave the lowest background values. Others have found that PBS plus 0.05% Tween 20 is sufficient for inhibiting nonspecific reactions (1). In our study, use of this diluent permitted 0.28 U of enzyme conjugate to adsorb to polystyrene cuvettes, which is as high a number as some we obtained in our positive tests. We had also found in a previous study on rickettsial antibody (8) that a combination of BSA and Tween 20 gave the best results, as have others. However, the disadvantage of using diluents with inhibitors is that desorption of antigen and antibody during an immunoassay is increased. We found that, for adsorbed viral antigen, a high percentage of virus elutes when it is incubated with the diluents used for immunoassay. Desorption of adsorbed antibody was minimal at concentrations of 2 and 10 μg of antibody protein per ml but was about 40% at 100 μg/ml. Engvall et al. (2), however, found that desorption of antibody was about 40% for antibody adsorbed at 2 μg/ml and incubated with PBS plus 0.05% Tween 20 alone, so that it would appear that optimal conditions for a given assay must be determined, in part, on an individual basis until a standard method is developed.

For enterovirus identification, the adsorbed antigen method was found to be approximately as sensitive as the adsorbed antibody method. Both methods are satisfactory, and, as the adsorbed antibody method has been used for detection of herpesvirus at low concentrations (14), it is more suitable as a virus detection method, especially if specific antisera of high titer from two species of animals are available. Although there is also a problem of antibody desorption, as discussed above, the advantage for virus assay is that virus can be tested without purification if the typing sera used does not contain antibody to antigens of the cells used for virus propagation.

The sensitivity of the enzyme-linked immunoassay method, in terms of virus concentration required for identification, was approximately 10^5 PFU of virus per assay tube. This is 10^2 to 10^3 times higher than the amount of virus required for identification by neutralization tests, but because the quantity of virus needed for the enzyme-linked immunoassay is readily obtainable in fluids from infected tissue cultures, this is not considered to be a serious disadvantage of the method. Also, virus titration is not necessary for the enzyme-linked immunoassay, which is an advantage over neutralization tests. The dilution endpoint of antisera that could effectively be used for the enzyme-linked immunoassay was approximately that obtained by neutralization tests.

In the work reported here we used individual typing sera to identify selected enterovirus types for developmental purposes. At the present stage of development, the method would be useful for presumptive identification of group B coxsackieviruses in situations where clinical or other evidence suggests involvement of these viruses,

as an alternate means of confirming a type-specific identification of an enterovirus isolate, or as a means to verify the identities of laboratory strains. If the method is to be useful for identification of field isolates, which could be any of a large number of enterovirus types, the use of pooled sera in an intersecting serum scheme (13) would be needed for rapid typing by the enzyme-linked immunoassay. Preliminary data indicate that some virus types can be identified by use of pooled sera, whereas others cannot due to cross-reaction, which is a current limitation of the method. The reasons for these cross-reactions and possible means to eliminate them are being investigated.

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