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Enzyme-Linked Immunosorbent Assays for Cholera Serology

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The enzyme-linked immunosorbent assay (ELISA) principle of Engvall and Perlmann, which employs antigen-coated tubes and enzyme-labeled anti-immunoglobulins, was elaborated for use in cholera serology. Immunoglobulin class-specific determinations of primary binding titers of antibodies to cholera exotoxin and endotoxin were more sensitive than were neutralization and vibriocidal tests. It was notable that rather high ELISA titers of immunoglobulin M (IgM) antibodies to exotoxin, which lacked neutralizing capacity were registered, whereas corresponding levels of immunoglobulin G (IgG) antibodies were effectively neutralizing. A modified technique permitting measurement of the binding rate of antibody to the solid-phase antigen was introduced as a possible tool for antibody avidity estimation. Such measurements indicated a wide avidity difference between the IgG and the IgM anti-exotoxin antibodies, which could explain their different neutralizing capacities. The observation that increasing binding rate of the IgG antibodies during the course of the primary immune response could compensate for decreasing antibody amount with regard to neutralizing capacity of serum also indicated the importance of antibody avidity for toxin neutralization. Inhibition with soluble antigen permitted quantitative determination of antigen; levels of exotoxin 0.09 μg/ml and of endotoxin to 1.3 μg/ml were measured.

Sensitive in vitro methods for serological study of microbial antigens are needed. This is especially true with the bacterial exotoxins including the diarrheogenic enterotoxin from *Vibrio cholerae*. Methods for measuring primary binding, rather than secondary, manifestations of the antigen-antibody reaction are preferable since they would overcome errors caused by the different capacity for secondary manifestations of the various immunoglobulin classes of antibodies (10, 11).

Recently, Engvall and Perlmann described an enzyme-linked immunosorbent assay (ELISA) for quantitation of antibodies to human serum albumin and 2,4-dinitrophenol (2). In the present study, we made use of this principle to elaborate methods permitting immunoglobulin class specific determinations of antibody titers and average antibody avidities to cholera exotoxin and endotoxin, as well as sensitive in vitro quantitation of these antigens.

MATERIALS AND METHODS

Antigens. Lyophilized, purified cholera exotoxin (choleragen) was supplied by National Institutes of Health (NIH), Bethesda, Md., and was prepared, as described (4), under contract for the National Institutes of Allergy and Infectious Diseases by R. A. Finkelstein. The University of Texas Southwestern Medical School, Dallas, Tex. Lipopolysaccharide (LPS) was prepared from *V. cholerae*, serotype Inaba (strain 35 A3), by the hot phenol-water extraction procedure followed by ultracentrifugation (9).

In addition, lyophilized culture filtrate (lot 4493G supplied by NIH; from *V. cholerae* Inaba strain 569 B and containing 1% LPS and 0.01% choleragen) and its 10-fold-purified fraction were used (5).

Antisera. Reference serum was prepared as follows. Three rabbits were given 12.5 mg of the 10-fold-purified culture filtrate fraction (10% LPS, 0.1% choleragen) in two subcutaneous injections 2 weeks apart. One week later they were bled, and the sera were pooled.

The same dose of this immunogen was also administered to three rabbits in a single subcutaneous injection, and serum samples were obtained 1, 2, 12, and 16 weeks after the injection. Corresponding samples from the animals were pooled and referred to as A, B, C, and D.

Fifteen rabbits were injected intravenously with Formalin-killed vibrios of strain 35 A3 in three, five-animal groups: (i) a single injection with 1×10^9 bacteria and the blood taken 10 days later (serum I),

(ii) three injections (2×10^8 bacteria each) given 2 weeks apart and the antiserum obtained from a bleeding 7 days after the last injection (serum II), and (iii) three injections (2×10^8, 3×10^7, and 1×10^7 bacterial, respectively) administered at the same intervals and the blood taken as in procedure (ii) (serum III).

In addition, a number of rabbit antisera taken from the primary, as well as the secondary, response against Formalin-killed 35 A3 bacteria or culture filtrate material were used.

Immunoglobulin fractions. Sera were filtered through an agarose A 1.5-m column which provided immunoglobulins M and G (IgM and IgG) in mutually uncontaminated form (7).

Anti-immunoglobulin sera. Goat antisera to rabbit IgG, immunoglobulin A (IgA), and IgM were purchased from Nordic, Tilburg, The Netherlands. The monospecificity was ascertained by immunodiffusion as well as by ELISA. The gamma-globulin fractions of these antisera were purified by repeated precipitation with half saturated ammonium sulfate.

Alkaline phosphatase. Alkaline phosphatase (specific activity 350 IU/mg), obtained from calf ileum mucosa, was purchased from Erco, Stockholm, Sweden.

ELISA. The technique described by Engvall and Perlmann (2) was employed with minor modifications. Alkaline phosphatase was conjugated to the gamma globulin fractions of anti-IgG, anti-IgA, or anti-IgM antisera; 1 mg of enzyme was used per 0.5 mg of gamma globulin, and the conjugation was effected by 0.2% glutaraldehyde at room temperature for 2 h. This procedure is simple compared to conjugation of only specific antibodies eluted from immunosorbent (2) but will, of course, result in lower activity since gamma globulin without the appropriate anti-immunoglobulin specificity will also be conjugated.

Antigen dissolved in phosphate-buffered saline (PBS; 0.15 M, pH 7.2) with 0.02% NaN_3 was coupled by mere incubation of 1-ml volumes at 37 C to the inner surface of disposable polystyrene tubes (11 by 70 mm; Nunc, Roskilde, Denmark). After removal of nonattached antigen by washing, the tubes were incubated, usually for 6 h, at room temperature in a roller drum (60 rpm) with 1 ml of antiserum dilution, the serum being diluted in PBS containing 0.05% Tween 20. Non-antigen-bound material was then washed off with PBS-Tween. Anti-immunoglobulin-enzyme conjugate (1 ml) at an enzyme concentration of 0.2 ng/ml was then allowed to react with antigen-bound antibodies of the appropriate immunoglobulin class; the tubes were thoroughly washed, and 1 ml of nitrophenylphosphate (1 mg/ml) was added as substrate. After suitable incubation time, the enzyme-substrate reaction was stopped by addition of 1 N NaOH, and the yellow color obtained was registered spectrophotometrically at 400 nm.

Neutralization test. The neutralization test of Craig (1) was used. The highest dilution of heat-inactivated serum which neutralized an equal volume of an exotoxin solution (40 blueing doses/ml) was recorded as the neutralizing antibody titer.

Vibriocidal tests. The spot agar plaque technique of Holmgren et al. (7) was utilized for titration of vibriocidal antibodies. Avidity estimation of these antibodies was performed by quantitative inhibition in a radial diffusion vibriocidal system (6).

Ammonium sulfate precipitation. The ammonium sulfate precipitation technique of Farr (3) was performed with ^{125}I-labeled lipopolysaccharide, and the avidity was calculated according to the Sips' distribution formula (6).

RESULTS

Preliminary experiments showed that cholera toxin (choleragen) and LPS from *V. cholerae* attached to polystyrene test tubes well enough to be used as antigens in the described assay. Optimal concentrations for coating of the test tubes were around 10 μg/ml for cholera toxin and 100 μg/ml for LPS (Fig. 1); the optimal incubation times were 3 and 6 h, respectively.

Testing of 10-fold serial dilutions of sera with the immunoglobulin class-specific enzyme conjugates permitted establishment of the type of curves shown in Fig. 2. With mixed anti-IgM- and anti-IgG-enzyme conjugates, intermediate curves were obtained which reflected the sum activity of the IgM and IgG antibodies. The highest serum dilutions which with the tested enzyme-conjugates gave significant extinction (chosen 0.15 above the generally negligible background level) were determined from the curves. The reference serum was included in each set of experiments to permit adjustment of results obtained in experiments performed on different occasions; the corrections were done in relation to the geometric mean from 10 experiments of the titer values of the reference serum. Antibody titers so determined had a reproducibility of standard deviation of 0.11 $\log_{10}$ units. In Fig. 3 the ELISA titers are compared with the neutralizing and vibriocidal antibody titers. On an average, 230-fold higher ELISA than neutralization titers and 23-fold higher ELISA than vibriocidal titers were registered, but considerable differences in titer ratios were noted with the various sera. Neutralization tests with separated IgG and IgM fractions showed that all of the neutralizing capacity of the antisera was due to IgG antibodies. In spite of this, IgM antibodies against cholera toxin were demonstrated with ELISA in titers up to 1:10,000 (Table 1). Against LPS, high IgG and IgM serum antibody titers were registered with ELISA, and both types of immunoglobulins were highly active in vibriocidal tests.

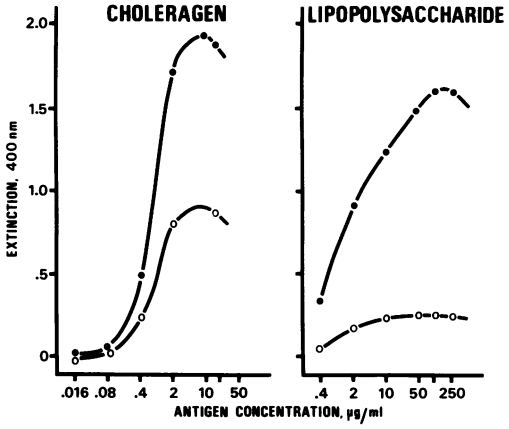

FIG. 1. *Effect of antigen concentration in the coating process. Coating was done for 3 h and evaluated with reference serum diluted 1:100 (●) or 1:10,000 (○) and with anti-IgG-enzyme conjugate.*

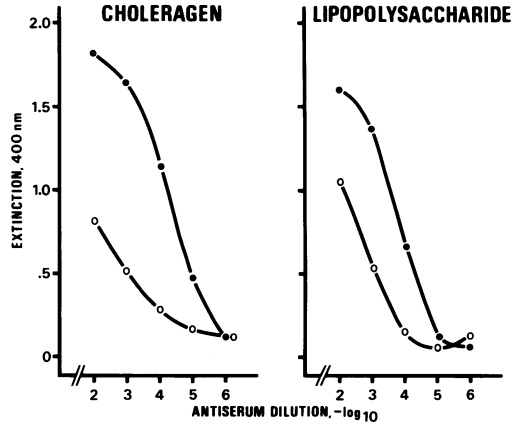

FIG. 2. *Specific antibody titration with ELISA with the reference serum and anti-IgG-(●) and anti-IgM-enzyme (○) conjugates. With the anti-IgA-enzyme conjugate, insignificant activity was found.*

Attempts were also made to inhibit the attachment of antibodies to the solid-phase (plastic-bound) antigen by adding different concentrations of soluble antigen to the antigen-coated tubes immediately before the incubation with antiserum. The effects obtained by inhibiting the reference serum with cholera toxin or LPS and by using the anti-IgG-enzyme conjugate are shown in Fig. 4. It was found that, with the 1:100 serum dilution employed, the inhibitory concentrations causing 50% reduction of the extinction (IC_{E50}) were 1.7 µg/ml for cholera toxin and 50 µg/ml for LPS. By increasing the serum dilution and preincubating it with the soluble antigen at +4 C overnight, the IC_{E50} values were reduced to 0.09 µg/ml for cholera toxin and 1.3 µg/ml for LPS. Parallel inhibition tests with purified exotoxin and LPS, and crude or only partially purified culture filtrate of *Vibrio cholerae* gave values for exotoxin and LPS contents which were in agreement with values previously obtained from neutralization-inhibition and vibriocidal-inhibition tests (8). The findings indicate that ELISA can be used for sensitive in vitro detection and quantitation of choleragen and LPS.

Inhibition experiments also revealed that, although ELISA is immunoglobulin class specific, antibodies of the nonassayed immunoglobulin classes might affect the results. This is illustrated in Fig. 5 where it is seen that, at concentrations of free LPS causing substantial inhibition of the IgG antibody activity, the attachment of IgM antibodies to the solid-phase antigen is increased instead. Similar results

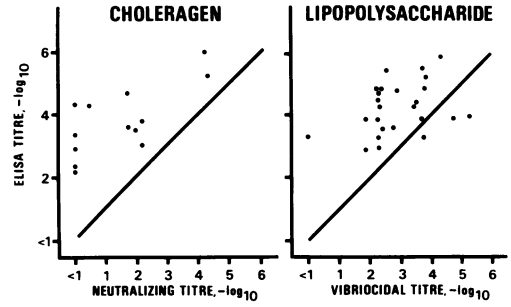

FIG. 3. *Comparison of titers obtained with ELISA and biological activity tests. The ELISA titers plotted are the sum of IgG and IgM titers registered.*

TABLE 1. *Primary binding properties in relation to neutralizing capacity of anti-choleragen antibodies from a primary immune response*

Time after immunization[a] (weeks)	Anti-choleragen titer (log_{10})				Binding time[b]	
	Neutralization[c]		ELISA			
	IgG	IgM	IgG	IgM	IgG	IgM
1	0	0	2.3	2.8	43	>720
2	1.7	0	4.7	3.7	23	440
12	2.1	0	3.8	<2	9	>720
16	2.2	0	3.0	<2	6	>720

[a] A single injection with crude toxin; sera A to D.
[b] Time in minutes required to obtain 50% maximal antibody activity.
[c] The serum titers are referred to as IgG since analyses of separated IgG and IgM fractions showed that all the neutralizing capacity resided in IgG.

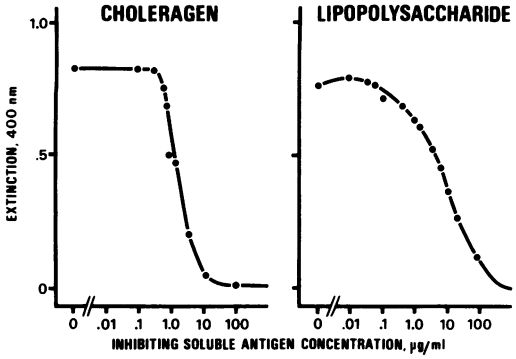

FIG. 4. *Quantitative inhibition with soluble antigen using the reference serum at dilution 1:100 and the anti-IgG-enzyme conjugate.*

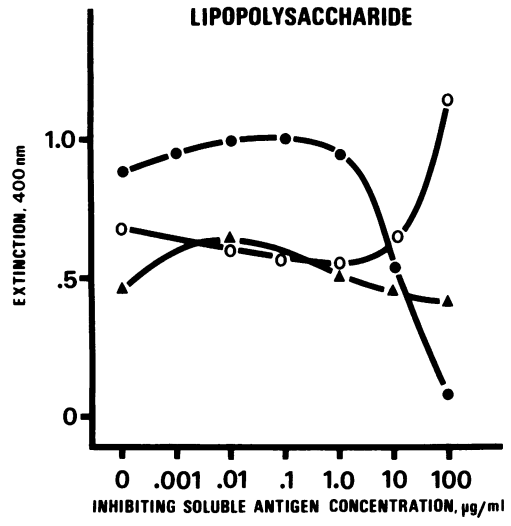

FIG. 5. *Influence of antibodies of nonassayed immunoglobulin classes studied by inhibition of anti-LPS antibodies with soluble LPS antigen. Tests were performed with anti-IgG-(●), anti-IgM-(○) and a 1:1 mixture of the anti-IgG- and -IgM-enzyme conjugates (▲) at serum concentrations fivefold those of the titers.*

were seen with all the sera tested, i.e., the reference serum and sera I, II, and III. Inhibition analyses of separated IgG and IgM fractions from these sera showed that 10 to 20 times more antigen was required for inhibition of the IgM than of the IgG antibodies of the same serum, probably reflecting different average avidities of the two antibody populations. The IgM antibodies also needed longer time than the IgG antibodies to attach to the solid-phase LPS antigen (Fig. 6a). It was also found that the binding to LPS of the IgG antibodies in sera I, II, and III occurred at a rate which corresponded to different avidities of these sera and their IgG fractions (I < II < III) as measured by the vibriocidal inhibition and the ammonium sulfate precipitation methods. Also, increasing binding rates corresponded to decreasing IC_{E50} values for the IgG fractions of these sera.

The antigen-binding rate was also studied for anti-cholera toxin antibodies. For the IgG antibodies the rate was found to increase with the time after a primary immunization (Fig. 6b and Table 1) and correlated better to the neutralizing capacity of the serum sample than did the ELISA titer (Table 1). For the IgM antibodies, which had no neutralizing capacity, the antigen-binding rate was extremely slow (Table 1).

DISCUSSION

Since in the described ELISA the antigen is only physically attached to the solid phase, it is important to find optimal concentrations of antigen for coating to get a "saturated carrier" with a minimum of antigen release. This was obtained with about 10 µg per ml of cholera toxin and 100 µg per ml of LPS from *V. cholerae*. However, fivefold lower concentrations of both

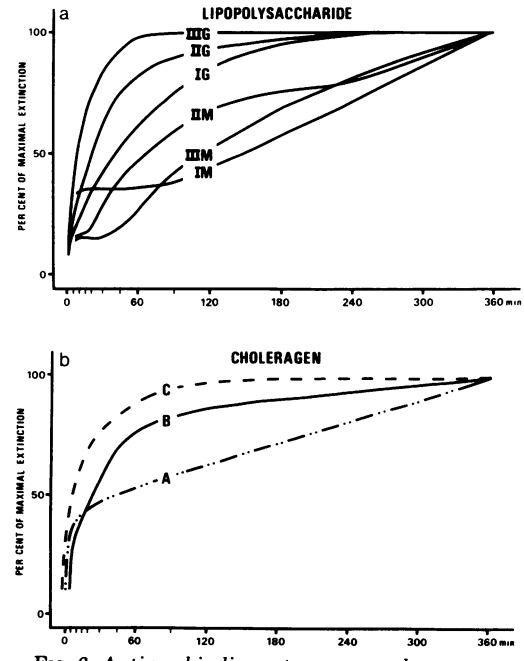

FIG. 6. *Antigen binding rates measured as percentage of maximal antibody activity obtained by incubation of antiserum for 5, 10, 15, 20, 30, 45, 60, and 90 min and for 2, 4, 6, and 24 h. The indicated sera were tested at concentrations fivefold the immunoglobulin class-specific antibody titers. a, Sera I to III tested with anti-IgG- and anti-IgM-enzyme conjugates; b, sera A to C tested with anti-IgG-enzyme conjugate.*

antigens gave 85 to 95% of an optimal extinction and were therefore chosen in the routine procedure to save material.

The conjugates of alkaline phosphatase and antibodies to IgG, IgM, or IgA made the registration of anti-toxin or anti-LPS antibodies immunoglobulin class specific. However, it seemed that antibodies of nonassayed immunoglobulin classes in an antiserum also competed for the available antigen determinants, which resulted in an underestimation of low avidity antibodies (mainly IgM) until antibodies of higher avidities (primarily IgG) had been functionally removed by the addition of soluble antigen. Thus, affinity-restricted or immunoglobulin class-specific immunosorbent removal of certain antibodies may facilitate detection of low-avidity antibodies.

The competition for antigen in ELISA between antibodies of different immunoglobulin classes may also make avidity estimation of sera by quantitative inhibition with soluble antigen (6) inaccurate. This problem might be overcome with the use of isolated immunoglobulins or immunosorbent-treated sera. An alternative approach to avidity estimations tried was to determine the antigen-binding rate of the antibodies. The rate seemed to be associated with the average antibody avidity, since increasing binding rates were registered with time after a primary immunization for anti-toxin (Table 1), as well as anti-LPS antibodies (unpublished data) when increasing avidities are expected. Moreover, in sera I, II, and III the binding rates to LPS corresponded to the average avidities estimated by the ammonium sulfate precipitation technique and by a quantitative inhibition method in agar plates (6).

The ELISA was also used for estimation of cholera exotoxin or LPS. The approach was quantitative inhibition with soluble antigen. Concentrations as low as 0.09 μg of exotoxin per ml and 1.3 μg of LPS per ml were quantitated, and the contents of these antigens in culture filtrate were determined. It is possible that ELISA would be useful for detection of the antigens in cholera stools.

The anti-toxin and anti-LPS titers registered with ELISA were higher than those measured by the intradermal neutralization test and the vibriocidal spot agar plaque method. The difference in titers obtained with ELISA and biological activity tests was most pronounced for anti-toxin antibodies in the beginning of the primary immune response. Only IgG antibodies showed any neutralizing capacity; in spite of this, substantial levels of IgM antibodies to exotoxin were revealed with ELISA. The superior neutralizing capacity of the IgG antibodies was probably due to their much higher avidity as estimated by antigen-binding rate measurements. The importance of affinity for neutralizing capacity is also indicated by the observation that, in spite of decreasing antibody levels registered with ELISA during the course of the primary immune response, the increasing antigen-binding rates noted were associated with rising neutralization titers.

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LITERATURE CITED

1. Craig, J. P. 1965. A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. Nature (London) **207**:614–616.
2. Engvall, E., and P. Perlmann. 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J. Immunol. **109**:129–135.
3. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I*BSA and antibody. J. Infect. Dis. **103**:239–264.
4. Finkelstein, R. A., and J. J. Lo Spalluto. 1970. Production of highly purified choleragen and choleragenoid. J. Infect. Dis. **121** (Suppl.):563–572.
5. Holmgren, J., I. Lönnroth, and Ö. Ouchterlony. 1971. Immunochemical studies of two cholera toxin-containing culture filtrate preparations of *Vibrio cholerae*. Infect. Immunity **3**:747–755.
6. Holmgren, J., and A.-M. Svennerholm. 1972. A radial diffusion agar plaque technique for estimation of the avidity of bactericidal antibodies against *V. cholerae*. Scand. J. Immunol. **1**:345–350.
7. Holmgren, J., A.-M. Svennerholm, and Ö. Ouchterlony. 1971. Quantitation of vibriocidal antibodies using agar plaque techniques. Acta Pathol. Microbiol. Scand. Section B. **79**:708–714.
8. Holmgren, J., A.-M. Svennerholm, and Ö. Ouchterlony. 1972. Experimental studies on cholera immunization. I. The response of neutralizing and vibriocidal antibodies in rabbits after immunization with culture filtrate material from *V. cholerae*. Acta Pathol. Microbiol. Scand. Section B. **80**:489–496.
9. Ørskov, F., I. Ørskov, B. Jann, K. Jann, E. Muller-Seitz, and O. Westphal. 1967. Immunochemistry of *Escherichia coli* O antigens. Acta Pathol. Microbiol. Scand. **71**:339–358.
10. Robbins, J. B. 1965. Studies on the interaction of immunoglobulins towards protein antigens with biological activity, p. 241–251. *In* J. Sterzl and coworkers (ed.), Molecular and cellular basis of antibody formation. Academic Press Inc., New York.
11. Robbins, J. B., B. Kenny, and E. Suter. 1965. The isolation and biological activities of rabbit γM- and γG-anti *Salmonella typhimurium* antibodies. J. Exp. Med. **122**:385–402.