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Comparison of Diffusion-in-Gel Enzyme-Linked Immunosorbent Assay with Conventional Serological Methods for Detection of Class-Specific Antibodies to *Salmonella typhi* O Antigen

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The diffusion-in-gel enzyme-linked immunosorbent assay (DIG-ELISA) is a new and simple method for quantitation of antibodies, based on the ability of antibodies to diffuse from wells in gel and adsorb to antigen which is bound to a polystyrene surface. The antigen-antibody reaction is visualized with a color reaction caused by horseradish peroxidase-conjugated class-specific anti-immunoglobins. This method was used to study the immunoglobulin G, A, and M immune response to *Salmonella typhi* O antigen in individuals immunized with a monovalent heat-inactivated typhoid vaccine. The antibody values obtained by the DIG-ELISA method correlated with those evaluated by conventional direct agglutination (Widal) and indirect hemagglutination methods. The DIG-ELISA method was also found to be sensitive, specific, and economical, as well as suitable for handling large numbers of sera while requiring very simple equipment.

The direct tube agglutination method (Widal) has primarily been used for determination of serum antibodies in cases of suspected typhoid fever (10, 13). Serum antibodies to *Salmonella* can also be determined with the indirect hemagglutination method (7, 11). The agglutination techniques only allow for determination of agglutinable antibodies and cannot discriminate between specific immunoglobulin G (IgG), IgA, and IgM antibodies.

Proteins, as well as other macromolecules of antigenic nature, adsorb to solid surfaces, forming a monomolecular layer and retaining antigenic activity (1). The antigen-coated solid phase is usually made of plastic and will act as an immunosorbent when exposed to the corresponding antibody. Visualization and quantitation of the antibodies bound can be performed by different methods. The most well known, the enzyme-linked immunosorbent assay (ELISA), was introduced by Engvall and Perlmann (5). With this technique, the amount of bound antibody is quantitated indirectly by the color intensity of the enzyme-conjugated anti-immunoglobulin bound to the specific antibody. Another method of evaluating the level of specific antibodies is to use their ability to form a diffusion gradient in a gel layered over an antigen-coated surface. The antigen-antibody reaction can then be visualized either by condensation of water (diffusion-in-gel thin-layer immunosorbent assay) (2) or by the color of an enzyme-conjugated anti-immunoglobulin (diffusion-in-gel [DIG]-ELISA) in an immobilized gel (4). Both of these methods allow for the detection of class-specific antibodies.

The present paper describes the application of the DIG-ELISA method for determination of specific serum antibodies to *Salmonella typhi* O antigen in individuals immunized with a typhoid vaccine. In addition, the DIG-ELISA method is compared with the direct and indirect hemagglutination techniques.

MATERIALS AND METHODS

Sera. Sera were obtained from 38 individuals before and up to 35 days after one immunization with a monovalent heat-inactivated typhoid vaccine (10^9 bacteria per ml; National Bacteriological Laboratory, Stockholm, Sweden). Some sera were also obtained from individuals after they had received a second injection. A total of 103 sera were analyzed. A more detailed report on the routes of immunization and doses has been given earlier (7).

Sera were also obtained from 40 healthy blood donors.

Antigen. *S. typhi* lipopolysaccharide (Difco Laboratories, Detroit, Mich.) was used as coating antigen in a concentration of 10 μg/ml.

Antibody determination of *S. typhi* O antibodies. (i) DIG-ELISA. The DIG technique for quantitation of antibodies and the antigen coating of polystyrene petri dishes (8.5-cm inner diameter; NUNC A/S, Roskilde, Denmark) has been described earlier

(3), as has the DIG-ELISA method with the horseradish peroxidase visualization system (4; H. Elwing, S. Lange, and H. Nygren, J. Immunol. Methods, in press). Briefly, the dishes were washed with 95% ethanol, dried and filled with 15 ml of 0.15 M NaCl containing 10 μg of *S. typhi* lipoplysaccharide per ml. After incubation for 60 min at room temperature, the dishes were washed three times with 0.15 M NaCl, after which 15 ml of 1% agar (Difco) in 0.15 M NaCl containing 1% horse serum was poured into each dish. When the agar had set, wells with a diameter of 3 mm were punched. The wells were filled with serum to the level of the agar surface, approximately 10 μl, and incubated in a moist atmosphere at room temperature for 24 h. The gel was removed by rinsing the dishes with phosphate-buffered saline (145 mM buffered to pH 7.1 with 70 mM phosphate)–0.05% Tween 20. The dishes were then incubated with phosphate-buffered saline-Tween for 5 min, followed by incubation in 10 ml of phosphate-buffered saline- Tween containing peroxidase-conjugated immunoglobulin (see below). After 2 h at room temperature, the dishes were rinsed three times with tap water and incubated with substrate gel. This gel consisted of 20 ml of 1% agar in 0.15 M NaCl and 10 mg of *p*-phenylenediamine in 1 ml of methanol and 0.01% H_2O_2; 10 ml was poured in each dish. The diameters of the DIG-ELISA reaction areas were read after 20 min, when the contrast between the black circular reaction area and the background was maximal. The zone diameter was measured with a ruler with an accuracy within ±0. 5 mm.

(ii) Direct agglutination and indirect hemagglutination methods. Serum antibodies to the *S. typhi* O antigen were determined by direct bacterial agglutination in tubes (Widal) (10) and with the indirect hemagglutination technique (9, 11). A Vi-negative *S. typhi* strain (NCTC 779) was used as the antigen.

Peroxidase-labeled conjugates. Horseradish peroxidase (type II, Sigma Chemical Co, St. Louis, Mo.) was conjugated to the immunoglobulin fraction of rabbit antisera specific for human IgG, IgA, and IgM (Meloy Laboratories, Springfield, Va.) as previously described (12). These conjugates can be stored lyophilized for at least 8 months without a decrease of the enzymatic activity, antigen-binding capacity, or alteration of the molecular weight as tested by sodium dodecyl sulfate electrophoresis.

Statistics. Regression analysis was used to evaluate the correlation between the different methods.

RESULTS

DIG-ELISA as compared to the direct agglutination (Widal) and indirect hemagglutination tests. The results in Table 1 show the correlation between the Widal titer values and the diameters of reaction area in DIG-ELISA. The correlation between these two test methods was highest for IgM (r = 0. 73) and IgA (r = 0.62) antibodies; a lower value was noted for IgG (r = 0. 48).

The results in Table 2 demonstrate the highest correlations between the indirect hemagglutination titer values and the diameters of the reaction area for antibodies of the IgM (r = 0.71) and IgA classes (r = 0.68); lower values were noted for IgG (r = 0.54).

TABLE 1. *Correlation between direct agglutination (Widal) titers and diameter of reaction area in DIG-ELISA*

Direct agglutination titer	No. of sera in each group	Reaction area (mm) in DIG-ELISA[a]		
		IgG	IgA	IgM
<20	30	7.4 ± 0.6	3.6 ± 0.2	4.1 ± 0.2
20	14	8.8 ± 0.7	5.7 ± 0.7	4.6 ± 0.4
40	17	9.3 ± 0.8	6.5 ± 0.6	6.1 ± 0.4
80	17	11.9 ± 0.5	7.0 ± 0.5	7.9 ± 0.4
160	15	12.7 ± 1.0	7.6 ± 0.7	8.1 ± 0.5
320	10	11.8 ± 1.2	7.1 ± 0.8	8.9 ± 0.8
Correlation coefficient (r)		0.48	0.62	0.73

[a] Mean ± standard error of the mean.

TABLE 2. *Correlation between indirect hemagglutination titers and diameter of reaction area in DIG-ELISA*

Indirect hemagglutination titer	No. of sera in each group	Reaction area (mm) in DIG-ELISA[a]		
		IgG	IgA	IgM
<2	1	6.0	≤3.0	≤3.0
2	11	7.0 ± 0.9	3.2 ± 0.2	4.1 ± 0.5
4	14	6.5 ± 0.6	3.8 ± 0.5	4.5 ± 0.6
8	22	9.3 ± 0.7	4.8 ± 0.6	5.6 ± 0.5
16	17	11.4 ± 0.6	5.8 ± 0.4	6.6 ± 0.4
32	13	13.2 ± 0.7	8.1 ± 0.5	7.6 ± 0.5
64	5	14.0 ± 1.2	8.4 ± 0.9	8.8 ± 0.4
128	9	13.2 ± 0.6	9.1 ± 0.6	9.6 ± 0.5
256	1	15.0	10.0	10.0
Correlation coefficient (r)		0.54	0.68	0.71

[a] Mean ± standard error of the mean.

***S. typhi* O antibodies before and after vaccination as determined with the DIG-ELISA method.** The results in Fig. 1 show that the diameter of the DIG-ELISA reaction increased significantly, i.e., more than 1 mm (Elwing et al., in press), in sera from individuals after one dose of typhoid vaccine. The diameter increase was significant for *S. typhi* antibodies of the IgG, IgA, and IgM classes. The diameter values before vaccination were higher for IgG (x = 7.5 ± 0.5 mm) than for IgA (x = 3.5 ± 0.2 mm) and IgM (x = 4.3 ± 0.2 mm). The prevaccination diameters for each immunoglobulin class of the vaccinees (Fig. 2) did not differ from the values found in 40 healthy

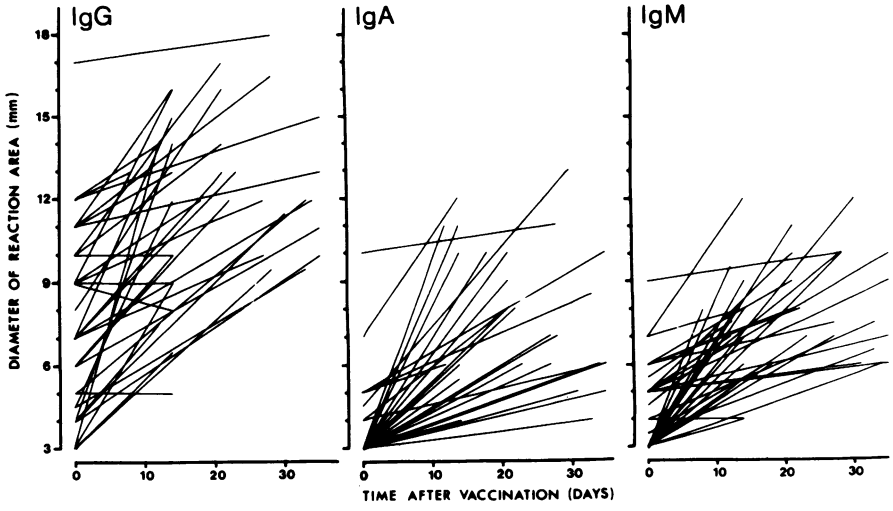

FIG. 1. *Serum antibodies of the IgG, IgA, and IgM classes to S. typhi O antigen determined with DIG-ELISA in 38 individuals before and after vaccination with one dose of typhoid vaccine. The DIG-ELISA values were recorded as diameter of reaction area (millimeters).*

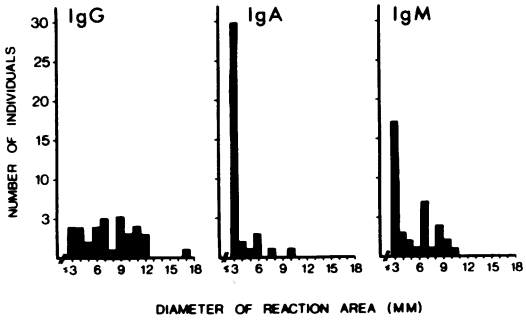

FIG. 2. *Prevaccination antibodies to S. typhi O antigen in sera from 38 individuals as measured with the DIG-ELISA.*

vaccines could not be shown to result in higher prevaccination serum antibody values. The prevaccination values could be reduced by 50% after one absorption of the sera (100 μl) with 50 μg of *S. typhi* lipoplysaccharide and were not measurable after absorption four times.

DISCUSSION

Serum antibodies to the *S. typhi* O antigen could easily be determined with the DIG-ELISA method, and the results could be correlated with those obtained by conventional direct and indirect hemagglutination techniques. It was also demonstrated that the zone diameters of the reaction area for IgG, IgA, and IgM increased significantly after one immunization with typhoid vaccine. In addition to direct and indirect hemagglutination tests, which only give information on the total amount of agglutinable antibodies, the DIG-ELISA method determines specific antibodies of different classes. It has been shown that the DIG-ELISA diameter is independent of the concentration of antigen during coating and that the diameter does not vary in size after development (Elwing et al., in press). These findings suggest that the antigen coating concentration, as well as the time for the reading of the reactions, is not critical, which is a methodological advantage in comparison to the conventional endpoint titer ELISA technique (6).

The prevaccination values, i.e., the diameter values registered before the immunization with typhoid vaccine, varied between the different immunoglobulin classes, with the highest values registered for IgG and the lowest for IgA. The prevaccination antibody values of the vaccinees and the antibody levels of the healthy blood donors showed similar values, suggesting that the vaccinated persons were representative of healthy individuals in general. These background values could be reduced by absorption with *S. typhi* lipoplysaccharides, indicating that the background values were caused by homologous or cross-reacting antibodies. Similar prevaccination titers have previously been found by using the agglutination tests (13). Whatever the type(s) of these antibodies might be, the existence of prevaccination titers indicates the necessity of analyzing at least two serum samples from each patient so that the variation of specific antibodies caused by illness or vaccination can be related to the initial level.

It has recently been shown that the peroxidase conjugates, as well as the substrate *p*-phenyl-

enediamine, can be lyophilized without loss of activity (H. Nygren, unpublished data). This permits easy storage of these components, which simplifies the performance of DIG-ELISA.

In our opinion, the results in the present paper and earlier findings (2, 4, 8) suggest that the DIG-ELISA method is an accurate and simple method for the detection of class-specific antibodies to the seven antigens so far tested (Elwing et al., in press). The method does not require highly purified antigens or titrations of antiserum, and the naked eye and a simple ruler are the only requirements for accurate registration of the results.

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