

Comparison of Enzyme-Linked Immunosorbent Assay and Passive Hemagglutination Method for Quantification of Antibodies to Lipopolysaccharide and Tetanus Toxoid in Rats

J. G. VOS, J. BUYS, J. G. HANSTEDDE, AND A. M. HAGENNAARS

National Institute of Public Health, Post Office Box 1, Bilthoven, The Netherlands

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In a comparative study, the enzyme-linked immunosorbent assay, using peroxidase labeled anti-rat immunoglobulin M and immunoglobulin G, and the passive hemagglutination test were applied to determine the primary and secondary antibody response to lipopolysaccharide and tetanus toxoid in rats. In the enzyme-linked immunosorbent assay, the antigens were bound to the wells of polystyrene microplates, tetanus toxoid directly, and lipopolysaccharide after complexing it with methylated bovine serum albumin. After incubation with dilutions of the rat sera, the amount of antibody bound to the solid phase was quantified by means of peroxidase-labeled anti-immunoglobulin. The specificity of the enzyme immunoassay was tested by absorption of the sera with their respective antigens. The enzyme-linked immunosorbent assay proved to be more sensitive than the hemagglutination reaction, except when titers were determined during the secondary response to tetanus toxoid. Besides its specificity and sensitivity, the enzyme-linked immunosorbent assay is a convenient method for measuring both immunoglobulin M and immunoglobulin G antibodies. At low serum dilutions of lipopolysaccharide antisera, inhibition of the reaction in the enzyme-linked immunosorbent assay occurred. This phenomenon could be prevented by heating the sera at 56°C for 30 min. Lipopolysaccharide was immunogenic in rats over an extremely wide dose range (from 10 pg to 1 mg); the optimal immunogenic dose of lipopolysaccharide for young adult rats was 0.1 to 1,000 µg when administered intravenously, and that of tetanus toxoid was 5 to 10 lines of flocculation, as determined by the Ramon flocculation test.

A routine method for the quantification of antibody titers is the indirect hemagglutination test (13). Recently, the enzyme-linked immunosorbent assay (ELISA) has been developed to measure antibodies, antigens, or haptens (4, 19).

This paper compares the quantification of antibodies in rats against *Escherichia coli* lipopolysaccharide (LPS) and tetanus toxoid by ELISA and the passive hemagglutination test. In the ELISA, antigen is coupled to a solid phase and incubated with antiserum, and the amount of antibody bound to the solid phase is quantified by peroxidase-labeled anti-immunoglobulin. As enzyme, most authors use horseradish peroxidase.

In mice, the antibody response against LPS does not require cooperation by T-helper cells (1), whereas both the immunoglobulin M (IgM) and IgG responses to tetanus toxoid are thymus dependent (18). Also in the rat, tetanus toxoid seems to be a thymus-dependent antigen, since pretreatment with antilymphocyte serum prevented a humoral immune response in this spe-

cies (E. J. Ruitenber and A. M. Hagenaars, unpublished data).

The advantages of ELISA are its sensitivity and its simplicity in detecting IgM and IgG antibodies. Because of the sensitivity, it is possible to measure antibodies against a weak antigen, such as LPS, in relatively poorly responding young rats. With tetanus toxoid as antigen, specific antibodies can be detected early after immunization. Habermann and Heller (7) described a sandwich and a competitive ELISA for the detection of tetanus antibodies, but these methods cannot discriminate between IgM and IgG antibodies, since peroxidase-coupled tetanus toxin was used as a tracer. Quantification of antibodies by ELISA against *Brucella* and *Yersinia* bacteria, using LPS as antigen, has been described by Carlsson et al. (3).

MATERIALS AND METHODS

Animals. Wistar-derived SPF rats were randomly bred in the Institute. The animals were housed in plastic cages and received food (Muracon, Trouw Ltd.,

Putten, The Netherlands) and water ad libitum. For the various experiments, approximately 8-week-old rats were used.

Antigens and immunizations. *Escherichia coli* LPS O127:B8, prepared by the phenol-extraction method (17), was obtained from Difco Laboratories (Detroit, Mich.). Tetanus toxoid, plain, was prepared in the Institute and contained 2.5 mg of protein per 1,000 lines of flocculation (Lf, as determined by the Ramon flocculation test).

Rats were immunized in the tail vein with different concentrations of LPS (1 µg to 1 mg) or tetanus toxoid (1 to 25 Lf). Blood was drawn from the orbital plexus on days 5 and 10. The second intravenous injection was given on day 10 with the same amount of antigen. The rats were bled from the heart on day 21. Sera were stored at -20°C until use.

Enzyme-conjugated anti-immunoglobulins. Anti-rat IgG was prepared by hyperimmunization of a sheep with purified IgG emulsified in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.). The antiserum to IgG (SaRaIgG) was rendered non-specific by absorption with F(ab')₂ fragments, obtained by peptic digestion of rat IgG (prepared by J. Nagel, Laboratory for Immunochimistry). Goat-anti-rat IgM (GaRaIgM) was obtained commercially from Nordic Pharmaceuticals and Diagnostics (Tilburg, The Netherlands). Immune globulin fractions from both antisera were prepared by caprylic acid fractionation by the method of Steinbuch and Audran (14).

The antisera against IgM and IgG appeared to be monospecific as tested by gel precipitation (Ouchterlony technique). In addition, the sera were tested for specificity in the more sensitive ELISA. For that purpose, wells of polystyrene microplates were coated with GaRaIgM or SaRaIgG, incubated with serially diluted normal rat serum, and finally incubated with peroxidase-labeled SaRaIgG and GaRaIgM. In this test system, the GaRaIgM appeared to contain less than 1% cross-reacting (anti-IgG) antibodies, whereas anti-IgM antibodies were virtually absent (<0.01%) in the SaRaIgG.

Horseradish peroxidase (PO), type VI, R.Z.3, was obtained commercially from Sigma Chemical Co., St. Louis, Mo. PO was conjugated to SaRaIgG and GaRaIgM by the method of Nakane and Kawaoi (9).

ELISA. Enzyme immunoassays were essentially performed as described by Ruitenberg et al. (10). Polystyrene microplates with eight rows of 12 flat-bottomed cups were coated with antigen by adding to each cup 100 µl of tetanus toxoid solution (2 Lf/ml) in sodium carbonate buffer (0.1 M; pH 9.6) with NaN₃ (0.02%). In trial studies, this concentration of tetanus toxoid gave optimal sensitivity. Because *E. coli* LPS alone poorly attached to polystyrene, after preliminary trials the following procedure was adopted. LPS was initially coupled to methylated bovine serum albumin (Miles Laboratories Inc., Slough, England). A mixture of 1 ml of LPS solution containing 0.5 mg of LPS in sodium carbonate buffer (0.1 M; pH 9.6) and 0.05 ml of methylated bovine serum albumin solution (10 mg/ml of distilled water) was incubated for 20 min at room temperature on a magnetic stirrer. For coating, the solution was diluted with carbonate buffer to a final concentration of 5 µg of LPS per ml (a concentration

that gave optimal sensitivity in trial studies). After coating for 2 h at 37°C, the microplates were washed with tap water of neutral pH containing 0.05% Tween 20. Serial twofold dilutions of the sera were prepared in phosphate-buffered saline (PBS; 0.01 M, pH 7.2) containing 2% bovine serum albumin and 0.05% Tween 20. Trays were incubated with 100 µl of the serum dilutions for 1 h at 37°C. After washing, 100 µl of conjugate, diluted to the proper dilution with PBS (0.01 M, pH 7.2), containing 2% bovine serum albumin and 0.05% Tween 20, was added. Microplates were incubated under rotation at 37°C for 2 h. The trays were washed again, and 100 µl of substrate was added. The substrate was prepared as follows. An 80-mg amount of 5-amino-salicylic acid was dissolved in 100 ml of distilled water at approximately 70°C. Directly before use, the pH of this stock solution was brought to 6.0 with 1 N NaOH. To 9 ml of this stock solution, 1 ml of 0.05% H₂O₂ was added. After incubation at room temperature, the reaction product was evaluated visually and expressed as 2_{\log} of the highest dilution, giving a positive reaction.

Control reactions included a negative serum control, using serum of nonimmunized rats, and a conjugate control (i.e., antigen-coated wells, to which conjugate was added and after proper incubation and washing, the substrate). In addition, absorption experiments were done. Sera were incubated at 37°C under rotation for 2 h with various concentrations of LPS and tetanus toxoid, stored overnight at 4°C, and centrifuged for 1 h at 20,000 rpm.

In one experiment, the antibody response to LPS was measured by the mechanized macro-ELISA (11, 12). For that purpose, disposable polystyrene tubes (50 by 11 mm; LKB-produkter AB, Stockholm, Sweden) were incubated with 1-ml quantities of the different reagents described above. Specific anti-LPS IgM antibodies were measured spectrophotometrically in twofold serially diluted sera.

Hemagglutination method. The hemagglutination assay for LPS was done largely by the method of Andersson and Blomgren (1), using sheep erythrocytes (SRBC) coated with LPS. LPS was dissolved in PBS (1 mg/ml) and boiled for 2 h while the pH was adjusted to 8 by adding 0.1 N NaOH. To 3 ml of boiled LPS solution, 1 ml of packed, washed SRBC was added and incubated for 45 min at 37°C. After this coating procedure, SRBC were washed three times in PBS and used for hemagglutination.

The hemagglutination method for tetanus toxoid was performed with Formalin-treated SRBC that were coated with tetanus toxoid with the aid of tannic acid. For that purpose, equal volumes of tannic acid solution (5 mg/100 ml of PBS) and 10% SRBC suspension in PBS were incubated at 37°C for 20 min. After washing in PBS, equal volumes of a 10% suspension of tannic acid-treated SRBC and tetanus solution (100 Lf/ml of PBS; pH 7.2) were incubated at 37°C for 20 min. SRBC were washed three times in PBS and used for hemagglutination.

Serial twofold dilutions (50 µl) of decemplemented (56°C for 30 min) sera in PBS with 0.5% BSA were prepared in the U-shaped wells of polystyrene microplates. A 50-µl amount of a 1% antigen-coated SRBC suspension was added. The plates were incubated for

1 h at 37°C. Results were expressed as 2_{\log} of the highest dilution giving positive agglutination.

RESULTS

Antibody response to LPS. The effect of different concentrations of LPS on the primary immune response as measured by ELISA is shown in Table 1. IgM antibodies were measured at day 5, since in preliminary studies the maximum IgM response was found 5 days after immunization. Antibodies of the IgG class were generally not detected in the primary response. LPS appeared immunogenic over a wide range of concentrations (from 10 pg to 1 mg). An antigen dose of 1 pg was not immunogenic, whereas a dose of 10 mg caused lethal endotoxin shock after intravenous injection. The optimal immunogenic dose of LPS in rats is 0.1 to 1,000 μ g.

The specificity of the ELISA is shown by the fact that after absorption of a positive serum with increasing amounts of LPS the 2_{\log} IgM antibody titer against LPS decreased from 11 to 2.

A comparison of ELISA IgM and the hemagglutination method is given in Table 2. Rats were immunized with LPS on days 0 and 10, and blood was collected at days 5, 10 (before the second injection), and 21. In all samples, the ELISA IgM titer was much higher than the hemagglutination titer. The IgM response was the same after 5 and 21 days. In contrast to day 5, antibodies of the IgG class were present on day 21 (data not presented).

A remarkable finding was that at low serum dilutions, the quantity of reaction product in the ELISA was lower than at higher dilutions. This inhibition was caused by a heat-labile serum component, as heating of the serum at 56°C for 30 min prevented this phenomenon (Fig. 1).

In Fig. 2, results are given of an experiment in

TABLE 1. Effect of different LPS doses on IgM antibody titers as measured by ELISA^a

Dose (pg/rat)	2_{\log} IgM titer
10^0	<0
10^1	5.3 ± 0.8
10^2	5.2 ± 1.9
10^3	6.8 ± 0.4
10^4	6.6 ± 0.5
10^5	7.7 ± 0.4
10^6	8.2 ± 1.0
10^7	7.4 ± 0.7
10^8	7.3 ± 1.5
10^9	7.4 ± 2.4

^a Four male rats were intravenously immunized. Values indicate results of the day 5 antibody titers, expressed as geometric means \pm standard deviation.

TABLE 2. Comparison of ELISA IgM and hemagglutination antibody titers during the primary and secondary response to LPS

Dose (μ g), days 0 and 10	Day after first immunization	2_{\log} titer ^a	
		ELISA IgM	Hemagglutination
100	5	6.4 ± 0.9	3.0 ± 1.2
	10	3.0 ± 0.7 (4)	1.4 ± 0.5
	21	6.3 ± 0.4	3.2 ± 0.8
300	5	6.0 ± 0.5	3.6 ± 0.8
	10	3.5 ± 0.5 (3)	2.2 ± 0.5
	21	6.2 ± 0.9	3.4 ± 0.5

^a Results are expressed as geometric means \pm standard deviation of five female rats per group. Values in parentheses indicate number of rats when deviating from five.

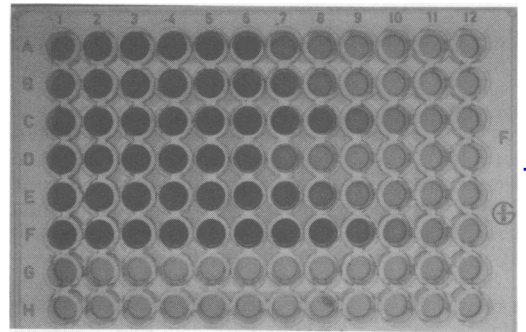


FIG. 1. ELISA for the demonstration of IgM antibodies to LPS, showing a reduction of reaction product at low serum dilutions in three positive sera, day 5 after immunization (rows A, B, and C). Rows D, E, and F: inhibition of the reaction is not present in these same sera after heating at 56°C for 30 min. Rows G and H are serum control and conjugate control, respectively.

which IgM antibodies to LPS are measured by the macro-ELISA. Twofold serial dilutions of the sera were tested and the extinctions measured spectrophotometrically. In the macro-ELISA, sigmoid curves were found that approached a linear function between the 2_{\log} serum dilutions of 4 to 7 or 8. These preliminary data indicate that, within that dilution range, specific antibody concentrations can be quantified spectrophotometrically by using one serum dilution.

Antibody response to tetanus toxoid. IgM and IgG antibodies to tetanus toxoid could be easily detected by ELISA. The assay is specific, since absorption of a positive serum with tetanus toxoid gave negative reactions.

The optimal immunogenic dose of tetanus toxoid in the rat when administered intravenously is 5 or 10 Lf (Fig. 3). Doses of 1 and 25 Lf did not induce detectable IgM antibodies at day

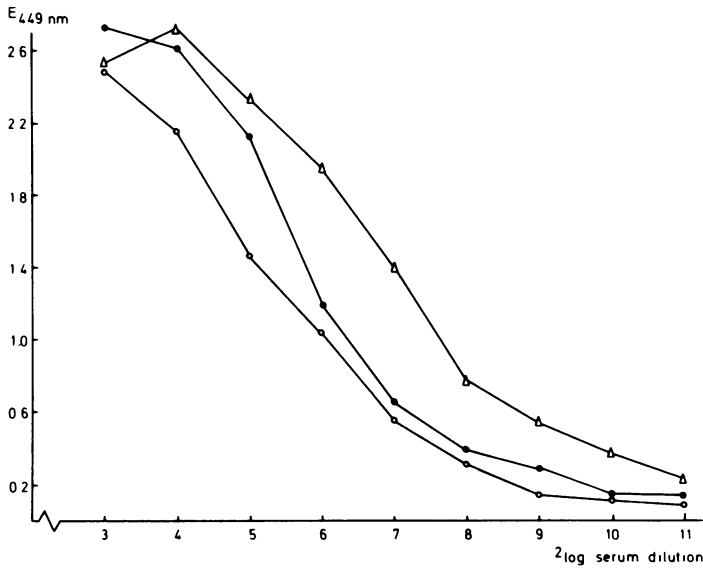


FIG. 2. Quantification of IgM antibodies against LPS by macro-ELISA. Sera of three rats were twofold serially diluted, and extinctions were measured spectrophotometrically. The sigmoid curves approach a linear function between the 2_{\log} serum dilutions of 4 to 7 or 8, thus demonstrating the possibility of quantifying specific antibody concentrations at a single reading within that region.

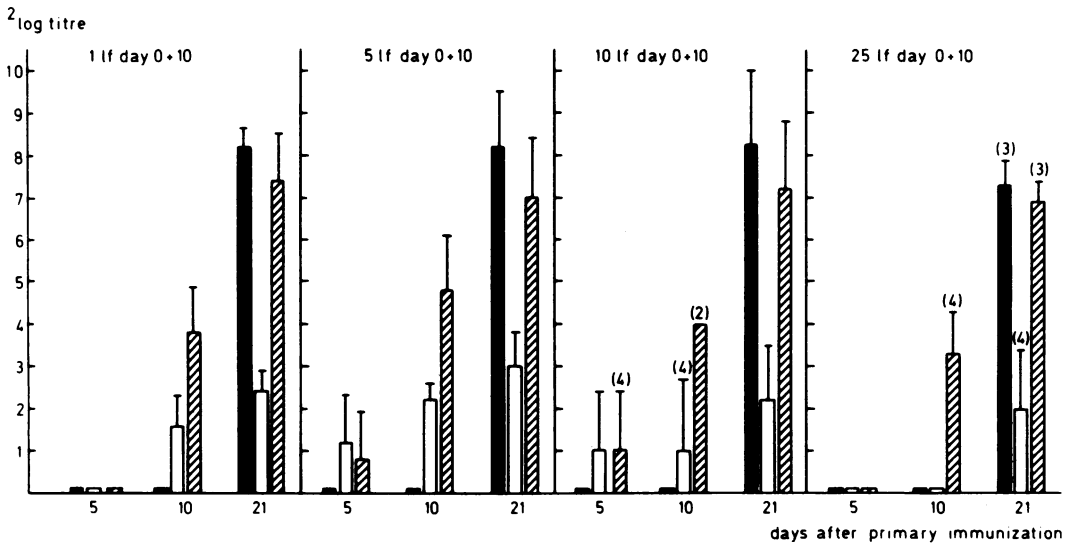


FIG. 3. Comparison of ELISA IgM (□) and IgG (▨) and hemagglutination (■) antibody titers during the primary and secondary response to tetanus toxoid. Results are expressed as geometric means \pm standard deviation of five female rats per group. Values in parentheses indicate number of animals when deviating from five.

5, as measured by ELISA. At day 10, antibody titers were higher, especially antibodies of the IgG class. At both day 5 and day 10, antibodies could not be detected by the hemagglutination method. In contrast, hemagglutinating antibodies were present at day 21, and might even be slightly higher when compared with the ELISA

IgG response (2_{\log} titers of 8 and 7, respectively). IgM antibodies were relatively low during the secondary response.

At low serum dilutions no inhibitions of the reaction, as seen in the LPS ELISA, occurred with the enzyme immunoassay for the measurement of antibodies against tetanus toxoid.

DISCUSSION

This study demonstrates that ELISA is a sensitive and specific method to determine antibodies against LPS and tetanus toxoid. The 2_{10G} titers of antibodies against LPS were approximately two times higher in the ELISA than in the hemagglutination method. By using ELISA, IgM and IgG antibodies could be easily determined during the primary immune response to tetanus toxoid, whereas the hemagglutination method did not detect antibodies at this time. Only during the secondary response to tetanus toxoid did the hemagglutination method prove successful, with titers that were higher than those obtained by ELISA, IgM, and IgG. Because IgM antibodies are very effective in the hemagglutination reaction, one would expect that the hemagglutination test would be more sensitive when predominantly IgM antibodies are present, e.g., against LPS. However, the reverse is true; the hemagglutination test was only a sensitive assay during the secondary response to tetanus toxoid, when high titers of IgG antibodies are found. Thus, the difference in sensitivity between both methods cannot be explained by the class of antibodies.

The sensitivity of ELISA is also demonstrated by the results of a study in which antibodies to LPS could be detected in suckling rats that were immunized as early as 1 day after birth (unpublished data). This is of particular importance for the study of possible immunosuppressive effects of chemicals, in which studies rats are preferably exposed during the ontogenesis of the immune system (15).

The accuracy and thus the discriminatory power of ELISA can possibly be further increased. This is of particular importance when subtle effects on the humoral immunity are studied. Preliminary results with the macro-ELISA showed that at certain dilutions of LPS antisera, extinctions were measured that approached a linear function if plotted against the dilution. In this way, antibody concentrations can be measured by a single reading, instead of by endpoint titration. Such an approach has been successfully applied for the quantitation of immunoglobulins, e.g., rabbit IgG (5) and recently rat IgM and IgG (J. G. Vos, F. M. H. Debets, J. Buys, and A. M. Hagenaars, manuscript in preparation). However, the slope of the curves found in the ELISA for total IgG was much less steep than the slope found in the present study. Also, Bullock and Walls (2) obtained a very steep slope in the ELISA for antibodies to *Toxoplasma gondii*, and they recommend to examine 2 or 3 dilutions to cover antibody levels that are mostly found.

In addition to the sensitivity of ELISA, a major advantage lies in the fact that specific antibodies of the IgM and IgG class can be easily determined. Measurement of IgG antibodies does not depend on prior degradation of IgM antibodies, e.g., by treatment with 2-mercaptoethanol, as is practiced for the hemagglutination method.

A remarkable finding in the present study was that, at low dilutions of LPS antisera, the reaction product in the ELISA was low when compared with higher dilutions. This inhibitory effect could be prevented by heating the sera at 56°C for 30 min. A thermolabile component of the complement system might cause this inhibition since bacterial LPS are known to interact with multiple complement components (6). Another possibility is the presence of a heat-labile LPS inactivator (8), a protein found in human serum that is capable of irreversibly disaggregating LPS. However, this inhibition seems to be a more general phenomenon, because it was also observed in titrating antibodies against *Corynebacterium parvum*, *Trichinella spiralis*, pneumococcal polysaccharide (SSS III), and ovalbumin (unpublished data).

The optimum immunogenic dose of tetanus toxoid for young adult rats found in this study is 5 or 10 Lf when administered intravenously, and that of LPS is 0.1 to 1,000 µg. However, LPS is immunogenic in rats over an extremely wide dose range (from 10 pg to 1 mg). Although not so pronounced as in rats, LPS is immunogenic in mice, also, over a rather wide range (16).

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