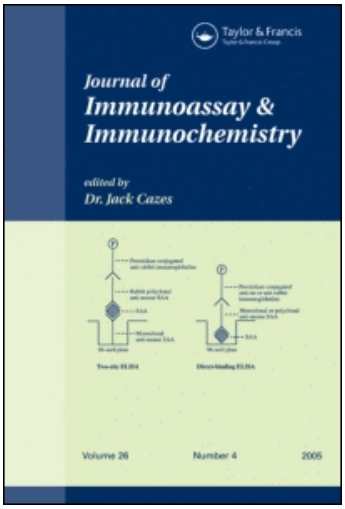

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Elisa for Human IgG and IpM Anti-Lipopolysaccharide Antibodies with Indirect Standardization

Anders Fomsgaard[a]; Bo Dinesen[b]
[a] Department of Infectious Diseases, Rigshospitalet, Copenhagen, Denmark [b] Nordisk Gentofte A/S, Gentofte, Denmark

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ELISA FOR HUMAN IgG AND IgM ANTI-LIPOPOLYSACCHARIDE ANTIBODIES WITH INDIRECT STANDARDIZATION

Anders Fomsgaard
Department of Infectious Diseases, Rigshospitalet,
Tagensvej 18, DK-2200 Copenhagen, Denmark.
and
Bo Dinesen
Nordisk Gentofte A/S, Niels Steensensvej 1, DK-2820
Gentofte, Denmark.

ABSTRACT

A new attempt to standardize ELISAs for the quantitation of human IgM and IgG anti-lipopolysaccharide antibodies (anti-LPS), without the use of a specific standard material, is described. Sandwich ELISAs for total IgG and IgM were combined with indirect ELISAs for anti-LPS IgG and IgM antibodies on a 96-well microtest plate using identical assay conditions. The concentration of specific IgG or IgM anti-LPS was read on the respective standard curve for total IgG or IgM. The results were corrected for residual immunoreactivity remaining unbound in the wells after one sample incubation in the combined assays. The quantitative results of IgG anti-LPS correlated well with results obtained using an ELISA with direct standardization (r=0.969). 28 mg/l of IgM anti-LPS was found as median value among 121 blood donors using the described ELISA principle. Binding studies demonstrated a lower apparent affinity of donor anti-LPS IgM than anti-IgG.

INTRODUCTION

Attempts to quantify specific antibodies are often made indirectly by the detection of marker reactions as a result of an

antigen-antibody reaction (hemolysis, hemagglutination, precipitation etc.). Many of these techniques do not distinguish between different immunoglobulin classes and may favor certain among them (1). ELISA for antibody is a highly sensitive method that measures total amounts of specific antibody and not just a functional subgroup (2). The method is able to quantify antibodies of different immunoglobulin classes, but the difficulties in, and the need for precise quantitations are reflected in the various ways results are presented: "end-point titre", "cut-off values", "absorption values", "activity units", "ratios" or the comparison to a "standard material", the concentration of which is measured by a different functional method (3). IgG and IgM antibodies to bacterial lipopolysaccharides (LPS) have different biological effects and may be of benefit in the treatment of patients in septic shock (1,4,5,6). However, the various ways of characterizing anti-LPS lead to different quantitative results (1,2). In order to circumvent the need for standard IgG and IgM anti-LPS antibodies, indirect methods as presented below were developed. This technique of indirect standardization for the quantitation of specific antibodies is proposed as a generally applicable method, whenever direct standardization is not possible or would not be accurate.

MATERIALS AND METHODS

Lipopolysaccharide Antigens

Smooth LPS extracted by the method of Westphal (Sigma Chemical Co., St. Louis, USA) was from eleven bacterial strains

and species (Escherichia coli O26:B6, E.coli O55:B5, E.coli O111:B4, E. coli O127:B8, E.coli O128:B12, Salmonella typhosa, S.typhimurium, S.enteritidis, S. Minnesota, S. abortus equi and Serratia marcescens). A mixture of these LPSs was used in the anti-LPS ELISAs.

Samples and Standards

133 plasma samples were taken from male and female blood donors, whose ages ranged from 18 to 65 years, and assayed in the IgG - and IgM anti-LPS ELISAs. A stable serum of human origin (Seronorm-103, Nycomed, Oslo, Norway) was used as a control in the assays for total IgG and IgM. A 1000-donor serum pool served four purposes: working standard in total IgG and total IgM assays (9.36 g/l of IgG and 1.05 g/l of IgM when substandardized against WHO Immunoglobulin Reference preparation 67/97); and control material for IgG- and IgM- indirect anti-LPS assays. A standard serum, containing 100 µg/ml of IgG antibodies to a similar mixture of smooth LPS as quantitated by an immunoprecipitation technique (6,7), was kindly provided by Dr S.L. Gaffin, Durban, South Africa, for comparison experiments.

Reagents and Buffers

Unconjugated and peroxidase-labelled rabbit anti-human antibodies came from Dakopatts, Copenhagen, DK (code A90, A91, P214 and P215). Buffer A: 0.1 M sodium hydrogen carbonate pH 9.8. Buffer B: 40 mM phosphate buffer, 0.1 M sodium chloride, pH 7.4

(PBS). Buffer C: phosphate buffer, 0.5 M sodium chloride, 0.1% Tween 20 (w/w). Buffer D: as buffer C with 1% rabbit normal serum (v/v) (code X902, Dakopatts, Copenhagen, DK). Buffer E: PBS with 0.5% bovine serum albumin, 0.1% Tween 20 (w/w). Washing solution: 0.15 M sodium chloride, 0.1% Tween 20 (w/w). Enzyme substrate: 12 mg o-phenylenediamine was dissolved in 15 ml distilled water and 6 µl hydrogenperoxide (30%) was added immediately before use.

Combined ELISA Design

Four ELISAs were modified so that they could be performed simultaneously and combined on one microtest plate: 1. A double antibody sandwich ELISA to measure total IgG (8). 2. An indirect method for assay of specific IgG anti-LPS (9). 3. An indirect method for assay of specific IgM anti-LPS (previously undescribed), and 4. A double antibody sandwich ELISA to measure total IgM (10). The combined design of these four ELISAs is described below. The ELISA steps are shown schematically in Fig.1 and the resulting picture in Fig. 2.

The incubation volume was 100 µl per well throughout the assay. The combined ELISA consisted of four individual steps (coating, plasma incubation, labelled antibody incubation and enzyme activity measurement). These four steps were separated by washing procedures. Coating: two columns to the left and two to the right of a 96-well polystyrene microtest plate (Teknunc, Roskilde, DK) were coated with rabbit anti-human IgG, diluted 1:5000, and rabbit anti-human IgM, diluted 1:16000 in buffer A,

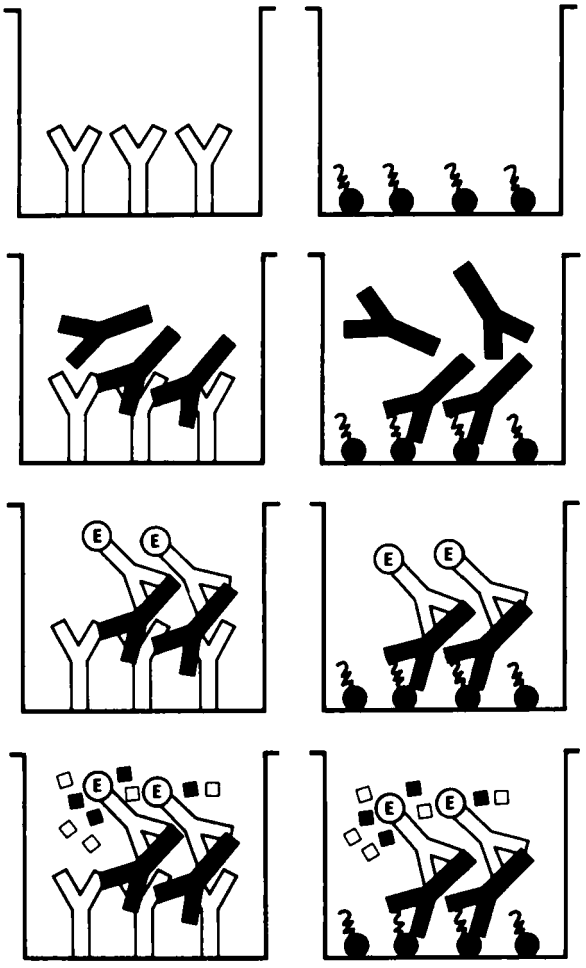

FIGURE 1. The 4 individual steps in the combined assay. The total amount of an immunoglobulin type is assayed in the left column and the specific antibody in the right. The same enzyme-labelled antibody is bound in the third step. Equal resulting enzyme activity is assumed to represent identical amounts of the assayed immunoglobulin type, whether bound to the solid phase through LPS (right) or through antibody (left).

respectively (Fig. 2). The remainder of the plate was coated with a pool of the eleven LPS (10 µg/ml of each) in buffer B. Coating proceeded at 4°C for at least 18 hrs and until use within one month. Washing: the plates were washed four times, using the Organon Teknika Washer, to separate the individual steps of the assay. Plasma incubation: test and control samples were diluted 1:100 and 1:500 in buffer D and pipetted into the left half of the LPS coated plate for the assay of IgG anti-LPS (columns 3-6). The same samples were diluted 1:200 and 1:500 in buffer E for the assay of IgM anti-LPS in the right half of the LPS coated plate (columns 7-10). Serial dilutions of the 1000-donor serum pool were pipetted in duplicate into the anti-IgG and -IgM coated wells. The IgG concentrations ranged from 4.68-93.6 µg/l of buffer D (columns 1-2), and the IgM concentrations from 5.25-105 µg/l of buffer E (columns 11-12). Overnight incubation (18 hrs) was at room temperature. Labelled antibody incubation: after washing, peroxidase-labelled rabbit anti-human IgG was diluted 1:15000 in buffer C and put into all the wells in the IgG/anti-LPS IgG half of the plate (columns 1-6). Peroxidase-labelled anti-human IgM was diluted 1:2000 in buffer E and put in all wells in the remainder of the plate. Incubation was at room temperature for 1 hr. Measurement of enzyme activity: after washing, enzyme substrate was added and the plates were placed in the dark for 30 min. The enzyme reaction was terminated by the addition of 150 µl 2.5 M H_2SO_4 to each well. The color in the wells was read at 486 nm with a 620 nm reference (A 486/620),

using a microtest plate photometer (Kontron SLT210, Zürich, Switzerland). The amount of specific IgG anti-LPS in the test samples was read on the simultaneously performed standard curve for total IgG. The amount of specific IgM anti-LPS was read on the simultaneously performed standard curve for total IgM.

To measure residual immunoreactivity in the samples after one completed plasma incubation, the contents of each well were transferred to an identically coated plate, and re-assayed. The ratio between A 486/620 obtained on the second and on the first plate, respectively, was calculated for each well. This ratio was used as an estimate of the individual residual immunoreactivity. The ratios obtained on the anti-immunoglobulin coated parts of the plate were much smaller than the ones obtained on the LPS-coated parts. Therefore, the results obtained (reading of the total immunoglobulin standard curves) would be underestimated if uncorrected for residual immunoreactivity. The correction employed was a multiplication with an experimentally determined factor: $F = \frac{1 - A^{II}s/A^{I}s}{1 - A^{II}t/A^{I}t}$, where $A^{I}s$ = absorption values obtained for total immunoglobulins in the first incubation; $A^{II}s$ = absorption values obtained for total immunoglobulins in the second incubation; $A^{I}t$ = absorption values obtained for specific anti-LPS in the first incubation; $A^{II}t$= absorption values obtained for specific anti-LPS in the second incubation. An example of the calculation for an individual sample is shown in the Appendix.

As a standard of comparison, 108 serum samples were also assayed for IgG anti-LPS concentrations, using the previously described ELISA with direct standardization (9).

RESULTS

The immunological reactions of the combined ELISA method are shown schematically in Fig 1. Immunoglobulin molecules, irrespective of their specificity, were bound by the anti-human type specific immunoglobulin solid-phase binder on the left. A standard serum containing a known amount of the assayed immunoglobulin type was used for calibration. On the right, the anti-LPS molecules were bound to the solid-phase bound LPS. The same conjugate was used simultaneously to detect bound immunoglobulin molecules in both assays. Equal enzyme activity on both sides is assumed to represent identical amounts of the assayed immunoglobulin type. Anti-LPS concentrations were therefore read on the standard curves for total IgG or IgM.

The use of a combination of all 4 ELISAs, performed simultaneously on one microtest plate, allowed for the quantitation of both IgG- and IgM anti-LPS in 16 samples, including one control sample per plate as shown in Fig. 2.

The specificity of the anti-LPS ELISA was demonstrated previously by absorption experiments (9). Optimal antibody concentrations in the ELISAs for total immunoglobulins were found using

the principles previously described (10). The dose-response curves are shown in Fig. 3. All samples were applied in two dilutions to confirm approximate parallelism with the IgG or the IgM standard curves (a 10% deviation was accepted). Sera with high anti-LPS concentrations (A above 1.800) were further diluted (10 fold) and assayed again.

The kinetics of the antibody-binding in the 4 ELISA methods were investigated by increasing the duration of the serum incubation (Fig. 4). Serum incubations seemed sufficiently complete within 2 hours, but an overnight incubation was selected for practical reasons.

The ratios (A^{II}/A^{I}) are believed to be measures of affinity of the different immunological reactions as occurring on the four different parts of the microtest plate. Median values for A^{II}/A^{I} in percent are shown in Table 1. An experimentally found correction factor had to be introduced to avoid an underestimation of the anti-LPS concentrations. For IgG anti-LPS, this factor was 2.1 (1.4 to 4.3)(median, central 95% interval, n=133). The corresponding factor for IgM anti-LPS was 3.1 (1.7-6.0) (median, central 95 % interval, n=121).

Several attempts were made to equalize apparent antibody affinities: different serum incubation temperatures (4°C, 22°C, 37°C and 50°C)(11) were combined with variation of the incubation time (15 min. to 24 hrs); the concentration of coating antibodies was varied; PEG 6000 (Merck, Darmstadt, FRG) was added to the serum incubation buffer; or incubation under

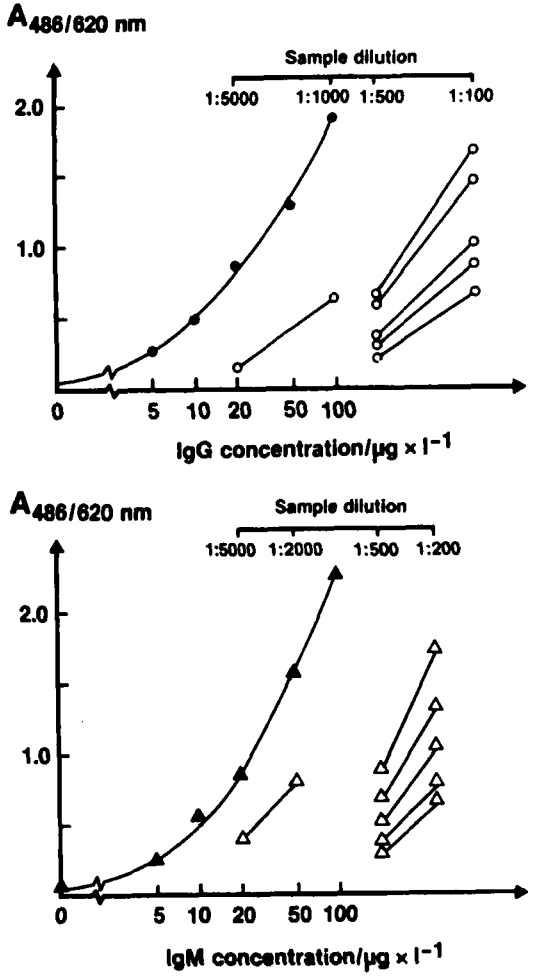

FIGURE 3. Top: IgG standard curve (●) and 6 sera (o) assayed in two dilutions (upper scale) for anti-LPS IgG (range 3-28 mg/l, correction factor 2.1). Bottom: IgM standard curve (▲) and 6 sera assayed in two dilutions (△)(upper scale) for anti-LPS IgM (range 10-120 mg/l, correction factor 3.1). The upper scales are arbitrarily placed.

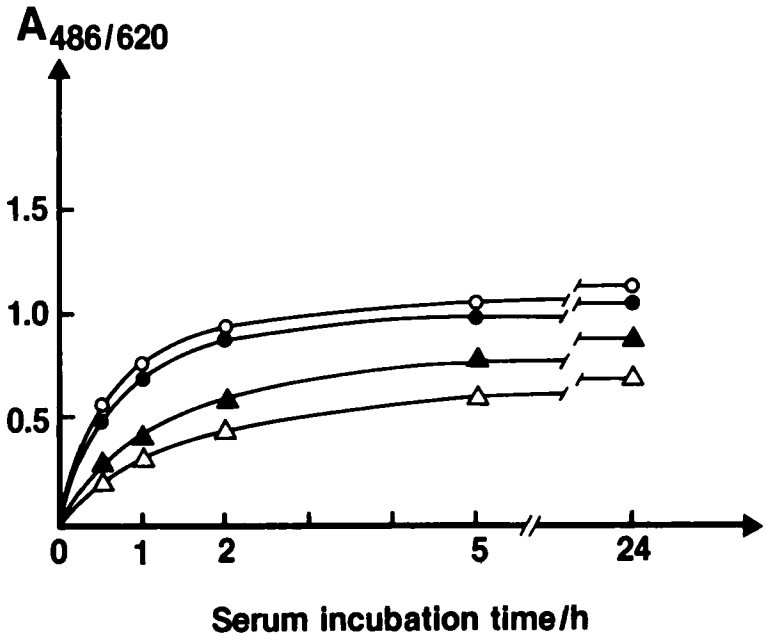

FIGURE 4. Duration of serum incubation. A 1000-donor serum pool was diluted 1:100, $1:2x10^5$, $1:10^4$ and 1:500, and assayed after incubations at different intervals for IgG-anti-LPS (o), IgG (●), IgM (▲) and IgM-anti-LPS (△), respectively. Equilibrium was approached sufficiently after 2 hours of incubation.

TABLE 1

PERCENTAGE OF TOTAL ANTIBODY-IMMUNOREACTIVITY BOUND AFTER COMPLETION OF THE FIRST SERUM INCUBATION IN THE 4 ELISAs.

	IgG	anti-LPS IgG	anti-LPS IgM	IgM
median	92%	44%	25%	77%
95% interval	-	22-65%	11-45%	-
range	88-98%	-	-	70-86%
	(n=56)	(n=133)	(n=121)	(n=56)

ultrasound (12) was tried. All these attempts failed to change the functional affinity of the antibodies. Thus, in order not to underestimate antibody concentrations, the introduced multiplication with an experimentally determined factor seemed unavoidable.

The median anti-LPS concentrations of the blood donors were 12 mg IgG per l (central 95% interval: 3-53 mg/l, n=133) and 28 mg IgM per l (central 95% interval: 7-120 mg/l, n=121). The relative standard deviations within and between assays were 4% and 6%, respectively, for assay of anti-LPS IgM (range 7-62 mg/l, n=32). The corrected IgG anti-LPS concentrations of 108 donors correlated well with the results obtained by direct standardization using the IgG anti-LPS standard serum (r=0.969)(Fig.5). The intercept of the regression-line shown in Fig.5 reflects a minor difference between non-specific-binding in the IgG ELISA and in the anti-LPS IgG assay.

An example of the clinical use of the assay is shown in Fig.6. A patient experienced septic shock characterized by coma, high fever, blood pressure below 80 mmHg and central venous pressure above 12 cm H_2O, periferal vasoconstriction and metabolic acidosis. Eschericia coli was cultured from the blood, and endotoxinemia was found during the shock state. Apparently the patient had the capacity to clear the endotoxins. The simultaneous decline in anti-LPS antibody concentration suggests a participation of anti-LPS in this clearance.

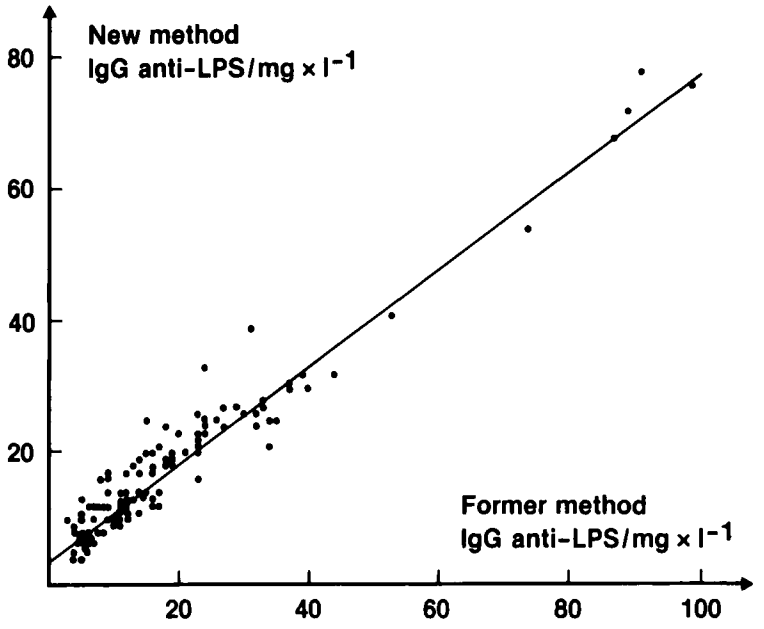

FIGURE 5. Comparison of IgG anti-LPS concentrations in 108 donor samples using the new quantitative combined ELISA (results are multiplied with the experimentally estimated median factor of 2.1) and direct standardization using the anti-LPS IgG standard as quantitated by immunoprecipitation (former method)(9). The regression line (——) was computed by means of linear least squares.

DISCUSSION

Specific antibodies to bacterial products including LPS are quantitated by different immunological methods, and the various ways of characterising such antibodies can lead to different quantitative results (1,2). Zollinger and Boslego (14) have suggested quantifying specific antibodies by a combination of two assays in a solid-phase radioimmunoassay (SPRIA). However, such

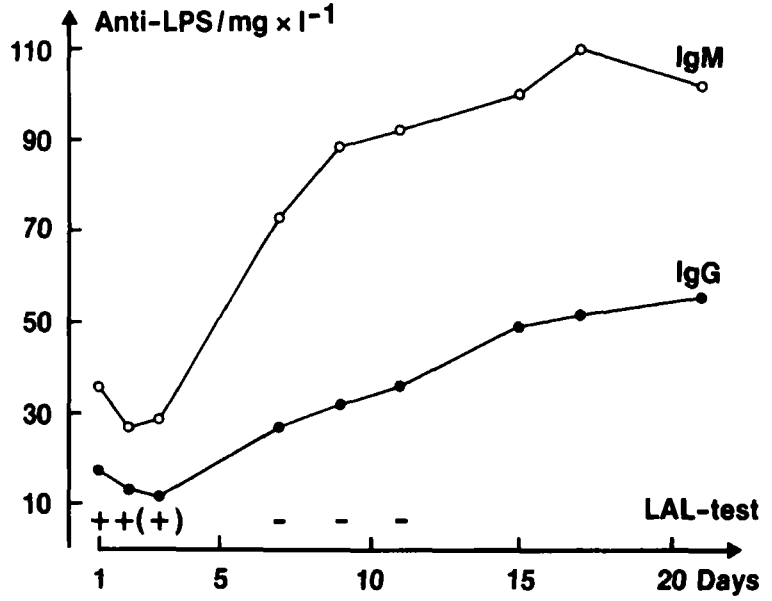

FIGURE 6. Plasma anti-LPS IgG (•) and -IgM (o) concentrations in a 77 year old man during and after a septic endotoxin shock. The patient had recovered from a pneumococcal meningitis 10 days earlier. No other underlying diseases were found. Septic shock: pyrexia (40°C), hypotension (mean arterial pressure <80 mmHg) and coma with E. coli grown from blood cultures and a positive Limulus amoebocyte lysate (LAL) test (13) developed at day 1. Declining anti-LPS antibodies were found during the acute illness. The patient was treated with antibiotics, dopamine infusion and respirator, and recovered on the 5th day. The septicemia was followed by a classical immunoresponse.

results may lead to less accurate estimations, when the affinity of the antibodies assayed differs from the affinity of the antibodies employed in reference assays.

The results of the present study demonstrate that human anti-LPS antibodies can be estimated by a combination of a sandwich ELISA for total immunoglobulin G and an indirect ELISA for anti-LPS IgG. However, it is necessary to correct for differences in

apparent affinity. The method was also used to quantitate anti-LPS IgM. By the simple transfer of serum incubation solutions to an identically coated solid-phase, and the measurement of residual immunoreactivity, the individual correction factors and a median factor can be determined (Appendix and Table 1).

The differences in remaining immunoreactivity (Table 1) may reflect differences in antibody affinity. The demonstrated difference between IgG- and IgM anti-LPS binding was found consistently under various assay conditions. This finding may reflect a lower affinity of anti-LPS IgM as compared to IgG in these healthy donors. Warren et al (15) found a lower concentration of IgM anti-LPS than of IgG anti-LPS in two pools consisting of 4 plasma units selected for high or low neutralizing effect on LPS in a Limulus amoebocyte lysate assay. Anti-LPS concentrations were estimated using the principle of Zollinger and Boslego (14). However, the antibodies measured were directed against single LPS preparations, making a direct comparison with our results difficult. The low IgM anti-LPS concentrations reported by Warren et al. might be explained by underestimation using the principle of indirect standardization without correction for apparent affinities.

Since anti-LPS antibodies have been shown to be beneficial in the treatment of Gram-negative bacterial sepsis (1,4,5), it is important to measure the concentration of different types of anti-LPS antibodies during sepsis. The assay presented here was used to measure anti-LPS antibodies in a patient under and after

septic endotoxin shock (Fig.6). A decline in both IgG and IgM anti-LPS antibodies was seen in the acute phase, probably caused by binding to endotoxin. During the convalescent phase a rise in anti-LPS IgM was accompanied by a slower rise in anti-LPS IgG. These increases in anti-LPS concentrations are believed to be due to immunization. Antibodies to the mixture of LPS antigens have been shown to cross-react with other species of Gram-negative bacteria (5). It can be assumed that anti-LPS antibodies bind to the endotoxic LPS in the circulation of the patient mainly by cross-reaction.

The advantage of the present method is its independence of specific standard materials. When both IgG and IgM anti-LPS antibodies are to be studied, the simultaneous performance of all 4 ELISAs on one microtest plate is convenient and less time-consuming. We therefore recommend this standard-free combined ELISA technique in the assay of human specific antibodies. This model can be used for the quantitation of different classes and subclasses of antibodies, whenever a direct standard material cannot be obtained.

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APPENDIX

Example of the calculation of the IgG anti-LPS concentration in a test sample using the indirect standardization method and an experimentally determined correction factor:

	A^I	A^{II}	A^{II}/A^I	A^{II}/A^I(mean)
ELISA for IgG				
Standard serum dilution				
$1:10^5$	2.357	0.189	0.080	
$1:2x10^5$	1.210	0.100	0.083	0.08
$1:5x10^5$	0.728	0.060	0.082	
ELISA for anti-LPS IgG				
sample dilution				
1:100	1.888	1.026	0.543	
1:500	0.896	0.509	0.568	0.55

$$F = \frac{1 - A^{II}s/A^Is}{1 - A^{II}t/A^It} = \frac{1-0.08}{1-0.55} = 2.04$$

Sample IgG anti-LPS concentration read directly on the standard curve for total IgG was 16.0 µg/ml. Corrected sample IgG anti-LPS concentration: 16.0 µg/ml x 2.04 = 33 µg/ml.