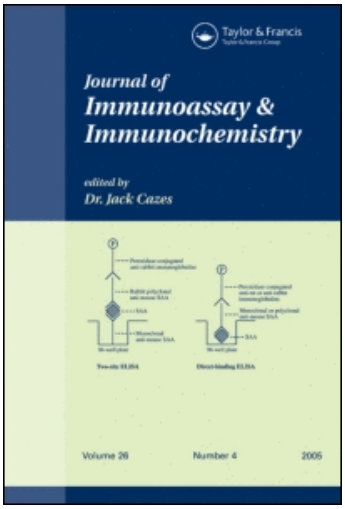

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Comparison of Blocking Agents for an Elisa for Lps

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To cite this Article Péterfi, Zoltán and Kocsis, BéLa(2000) 'Comparison of Blocking Agents for an Elisa for Lps', Journal of Immunoassay and Immunochemistry, 21: 4, 341 — 354
To link to this Article: DOI: 10.1080/01971520009349541
URL: http://dx.doi.org/10.1080/01971520009349541

JOURNAL OF IMMUNOASSAY, 21(4), 341 354 (2000)

COMPARISON OF BLOCKING AGENTS FOR AN ELISA FOR LPS

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ABSTRACT

ELISA is a sensitive, specific, reproducible and fast method for detection of antigen-antibody reactions. In case of non-protein antigens as LPS, problems exist, such as poor proportion of coating to microplates, non-specific binding of antibodies to the plastic wells. These problems were resolved partially by Takahashi and co-workers using poly-L-lysine for coating of LPS antigens. To reduce non-specific binding, blocking agent, such as bovine serum albumin (BSA) or casein is commonly used. We have to choose the blocking agent carefully because LPS can bind proteins non-specifically. This process can inhibit binding of LPS-specific antibody to LPS and decrease the sensitivity of method. In this paper we describe an ELISA test for LPS in which normal goat serum is used for blocking. This modification increases the sensitivity of ELISA. This method is useful for detection of LPS (S, R form) and anti-LPS antibody reaction in serological cross-reaction studies. (KEY WORDS: ELISA, goat serum, lipopolysaccharide, serological cross reaction)

INTRODUCTION

Enzyme-linked immunosorbent assay (ELISA) is probably the most frequently used method for estimation of antibodies. In the case of lipopolysaccharide (LPS) antigens their poor coating to microplate is problematic. Takahashi and co-

workers (1) described a good method for coating LPS antigens using poly-L-lysine for precoating. Another problem of ELISA is the non-specific binding of antibodies to the plastic wells. We have to choose the blocking agent carefully because LPS can bind proteins non-specifically. The present paper describes an ELISA test for LPS in which normal goat serum is used for blocking. This modification has several advantages, which are shown and discussed in this paper.

MATERIALS AND METHODS

Bacteria and antigens

Antigens were extracted from different types of *Shigella sonnei* mutants: *S. sonnei* phase I and II (4), *S. sonnei* Re 4350, respectively *Escherichia coli* O21 and O111, *Salmonella urbana* O30, *S. adelaide* O35, *Yersinia enterocolitica* O9, *Proteus morganii* O1, O9 and O43.

Bacteria were grown in fermentor (Braun Melsungen -Biostat U 30). The endotoxic lipopolysaccharides (LPS) were prepared from S-form bacteria by phenol-water method (5) and from R-form bacterium by phenol-chloroform-petrolether method (6). Lipid A was obtained by 1% acetic acid (100 °C, 90 min.) hydrolysis of LPS.

Production of antisera

Bacteria used for immunization were grown in agar medium at 37 °C overnight, washed and adjusted in saline to give 1 x 10^8 cfu/ml. Bacterial suspension was boiled at 100 °C for 1 h.

New Zealand rabbits (mean weight 3 kg) were immunized intravenously (7) with bacterial suspension: 0.2 ml (day 0), 0.4 ml (day 5), 0.8 ml (day 10), 1.2 ml (day 15), 1.6-ml day (20), 1.8 ml (day 25). Titres were controlled by tube agglutination and rabbits were bled. The serum was removed, filtered to sterilize and stored at -20 °C. Animals in this study were used in accordance with the University Medical School of Pécs Guidelines of Animal Experimentation.

Direct ELISA

The technique described by Engvall and Perlmann (8) and modified by Takahashi (1) was used as the basis for the assay. Below are the steps used in our ELISA:

Precoating of microplates: A solution (100 µl) of poly-L-lysine (MW. 260 000) (Sigma Chemicals, St. Louis, MO, USA) (10 µg/ml) in 0.01 M phosphate-buffered saline at pH 7.2 (PBS) was placed in polystyrene microplates (Nunc Immunoplate, Intermed, Denmark). The solution was incubated overnight at room temperature.

Binding of LPS: Aliquots of various concentration of LPS (100 µl) suspended in PBS was placed in poly-L-lysine precoated plates and then incubated for 1 h at 37 °C. The plates were washed four times with PBS containing 0.05% Tween 20 (T-PBS).

Blocking of non-specific binding sites:

Blocking reagents: Casein, BSA, goat, sheep, pig, bovine, guinea pig, horse sera were purchased from Sigma Chemicals (St. Louis, MO, USA). Aliquots of

different dilution of 0.5% casein or BSA or sera (200 µl) were placed in each well, incubated for 30 min. at 37 °C and washed four times with T-PBS.

Antisera: An optimum dilution of rabbit immune serum (100 µl) was added to each well, incubated for 1 h at 37 °C and washed four times with T-PBS.

Conjugate: Aliquots (100 µl) of a 1/500 dilution of peroxidase conjugated anti-rabbit Ig G (goat sera) (Sigma Chemicals, St. Louis, MO, USA) were added to each well, incubated for 1 h at 37 °C and washed four times with T-PBS.

Substrate: The peroxidase substrate solution containing 0.01% o-phenylene diamine (Sigma Chemicals, St. Louis, MO, USA) and 0.03% H_2O_2 (100 µl) was added to each well. Reactions were stopped about 10 min. later by 4 N H_2SO_4. The optical density (OD) unit was read at 492 nm in Titertek Uniscan reader (Flow Laboratories, Helsinki, Finland).

Quality control

The reader was blanked on air and the wells were read. The OD of buffer control must be greater than 0.000 but less than 0.100. If the value was above 0.100, inadequate washing was considered and the experiments were repeated. The difference between the OD value of positive control and negative control (IgG off) must be at least 0.800. If this value was less then 0.800, we did not take results into consideration. After quality control the reader was blanked on the negative control.

The data presented represent the mean and standard errors of at least triplicate samples from three separate experiments. Student's t-test statistical difference was accepted for $p<0.05$ or $p<0.001$.

RESULTS

Optimization of LPS concentrations

Microplates were coated with various concentrations of *S. sonnei* phase I LPS. Good response was obtained with LPS concentrations higher than 0.1 μg/ml. (Figure1). For further experiments we used LPS concentration at 1 μg/ml.

Optimization of blocking

Microplates were coated with *S. sonnei* phase I LPS at 1 μg/ml. Non-specific binding sites were saturated with varying dilutions of casein (0.5%), BSA (0.5%) and sera (Figure 2). Minimum OD background was obtained with a serum dilution at 1/20 and a casein and BSA dilution at 1/100 respectively. We used these dilutions for further experiments.

Comparison of various blocking agents in ELISA

For good detection a low non-specific background and higher difference between OD values of positive and negative controls is necessary. Therefore, we looked for an optimum blocking agent with minimal non-specific background.

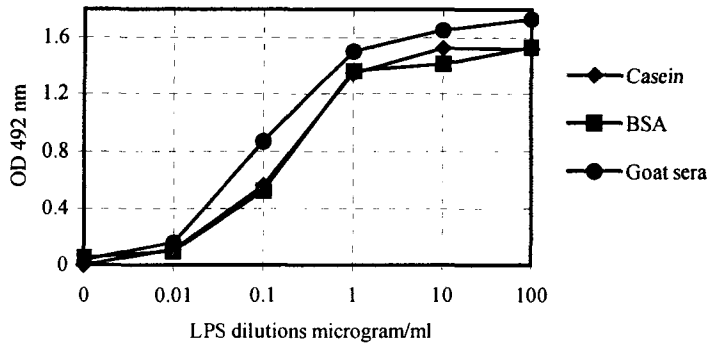

FIGURE 1: Optimization of LPS concentration: Poly-L-lysine precoated microplates were coated with various concentrations of *Shigella sonnei* phase I LPS, and treated with antisera. For blocking non-specific binding sites we used casein (♦), BSA (■), goat serum (●).The OD unit was read relative to the negative control.

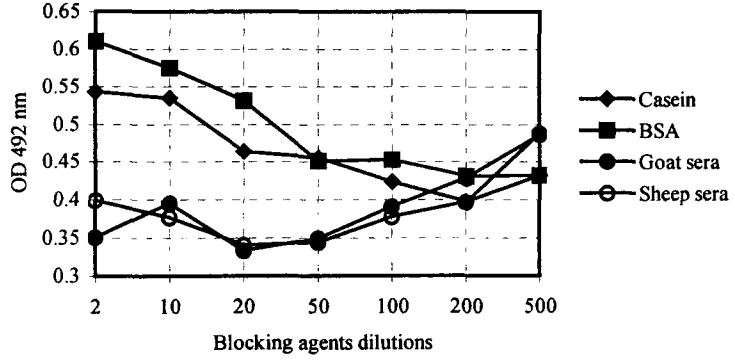

FIGURE 2: Optimization of blocking: Microplates were coated with *Shigella sonnei* phase I LPS. Non-specific binding sites were saturated with casein (♦), BSA (■), goat (●), sheep (○) sera. The OD unit was read with reader blanked on air.

Casein, BSA, goat, sheep, pig, bovine, guinea pig, horse sera were tested. The traditional casein and BSA were compared to animal sera. Goat serum gave statistically significantly lower OD value for negative control ($p<0.001$) and statistically significantly higher OD values for positive control ($p<0.001$). We could not find statistical differences between sera originated from different animals.

Precision data

Intra-assay variations were determined using 6 simultaneous determinations ($p<0.001$, SD 0.033 for positive controls, respectively $p<0.001$, SD 0.023 for negative controls) using goat serum in comparison to casein or BSA. Inter-assay variations were determined by testing 12 determinations performed individually ($p<0.001$, SD 0.088 for positive controls, respectively ($p<0.001$, SD 0.097 for negative controls) using goat serum in comparison to casein or BSA.

Reproducibility of data

It is well known there is individual variation in protein, immunoglobulin concentration of sera. For excluding this uncertainty we compared goat sera from 20 different animals. No significant variation was obtained (SD 0.044 for negative, respectively 0.080 for positive control).

Competitive reblocking test

In simultaneous tests non-specific binding sites were blocked, firstly with

casein, BSA and goat serum. In the next step, wells blocked with casein or BSA were re-blocked with goat serum, respectively. Wells blocked with goat serum were re-blocked with casein or BSA. When goat serum is used for re-blocking, the results were significantly better ($p<0.05$) than when casein or BSA were used alone. If casein or BSA were used for re-blocking, the results were significantly worse ($p<0.05$) than when goat serum was used alone.

Comparison of various LPS forms

LPS extracted from different smooth and rough strains such as *S. sonnei* phase I and II, *S. sonnei* Re 4350, *Escherichia coli* O21 and O111, *Salmonella urbana* O30, *S. adelaide* O35, *Yersinia enterocolitica* O9, and *Proteus morganii* O1, O9 and O43 were used for testing the usefulness of our ELISA method. The results were similar for both smooth and rough strains (Figure 3). Goat serum is the best choice for blocking.

Cross reaction analysis of LPS

LPS structure of *Shigella sonnei* strains is well known and their immunological characteristics were studied earlier. *S. sonnei* phase I (9) is a wild strain, therefore its LPS contains lipid A-core-O specific chain. *S. sonnei* phase II LPS (10) does not contain O specific chain and *S. sonnei* Re 4350 LPS is an absolute rough mutant, containing lipid A-KDO.

In our experiment antisera to *S. sonnei* phase I cross react with *S. sonnei* phase II LPS, but it could not react to *S. sonnei* Re 4350 LPS. Antisera against *S. sonnei*

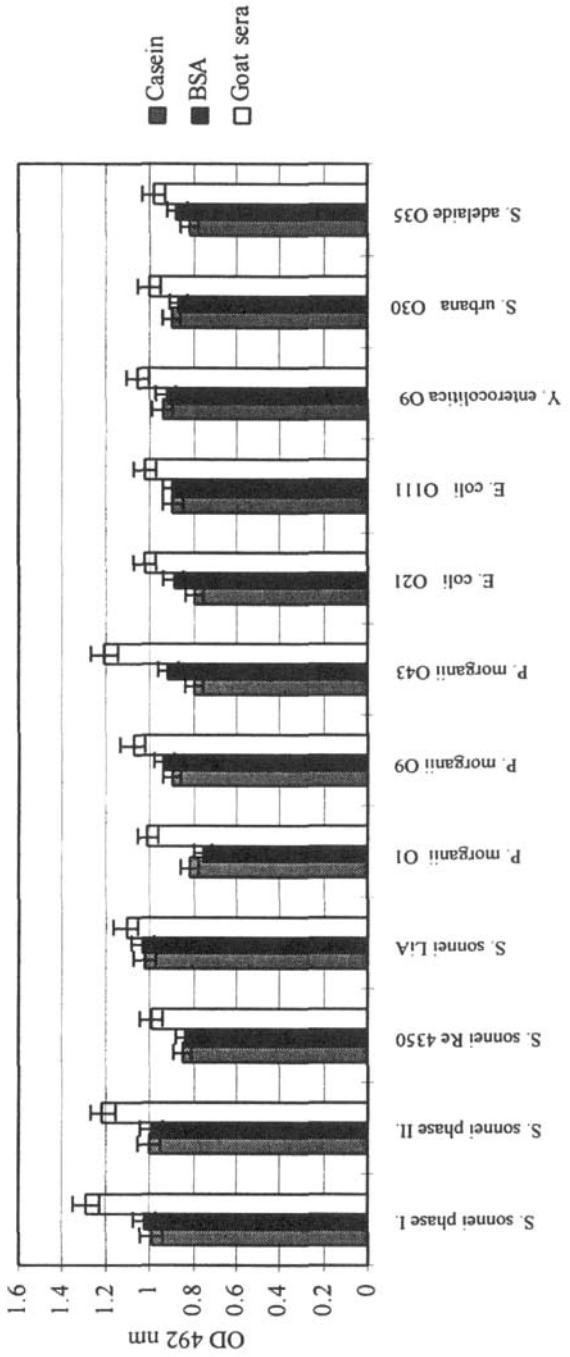

FIGURE 3: Comparison of various LPS forms using casein, BSA and goat serum for blocking non-specific binding sites: Microplates were coated with LPS extracted from different strains and treated with homologous antiserum. The OD unit was read relative to the negative control.

phase II and *S. sonnei* Re 4350 cross react to *S. sonnei* phase I, *S. sonnei* phase II, *S. sonnei* Re 4350 LPS, too, showing that these antigens contain common structural components and are capable to cause serological cross reaction. We compared the effect of the blocking agents on the sensitivity of the ELISA test. Our results show statistically significantly higher OD values when we used goat serum for blocking (Figure 4). In Figure 5 the same results are shown in other form. We subtracted the OD value of *S. sonnei* Re 4350 from S. *sonnei* phase I, OD value of *S. sonnei* phase II from *S. sonnei* phase I and OD value of *S. sonnei* Re 4350 from *S. sonnei* phase II. The differences are demonstrated in function of blocking agents: 0.5% casein, 0.5% BSA and 1/20 dilution of goat serum. As Figure 5 shows the ELISA test is most sensitive and suitable for cross reaction analysis when goat serum is used for blocking.

DISCUSSION

ELISA is a sensitive, specific, reproducible and fairly fast method. In case of non-protein antigens as LPS, problems exist, such as poor proportion of coating to microplates, and non-specific binding of antibodies to the plastic wells. The first problem was resolved by using poly-L-lysine for coating LPS antigens (1). Originally peroxidase conjugated Protein A was used in the test, but the maximum OD value obtained was not high enough (mean OD 0.751 in our experiment). If we used peroxidase conjugated anti Ig-G without blocking the background signal was too high (mean OD 0.946 in our experiment). Non-specific binding is usually reduced by blocking agents such as 0.5% casein or 0.5% BSA solution (2, 3). If

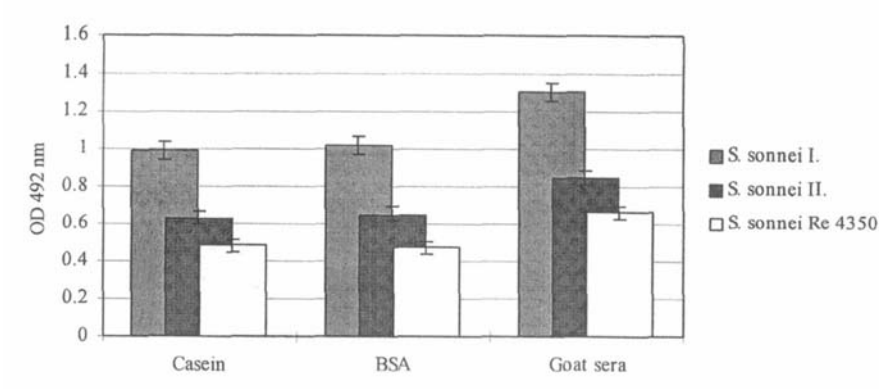

FIGURE 4: Cross reaction analysis: Microplates were coated with *Shigella sonnei* phase I. LPS and treated with *S. sonnei* phase I, *S. sonnei* phase II, *S. sonnei* Re 4350 antisera. We used 0.5 % casein, 0.5 % BSA and 1/20 goat serum for blocking. The OD unit was read relative to the negative control.

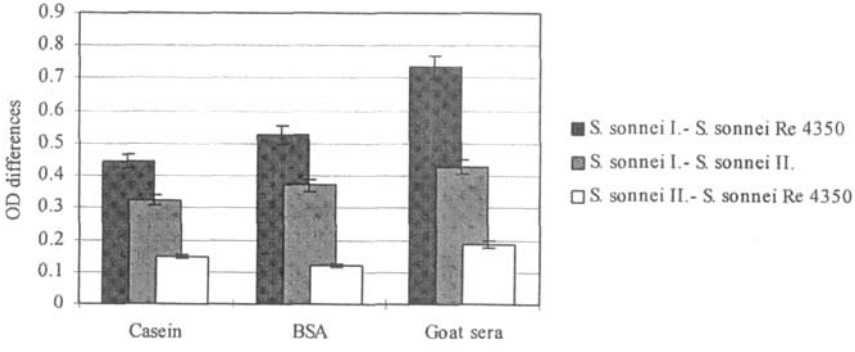

FIGURE 5: Cross reaction analysis: The procedure is given in FIGURE 4. Difference between OD values for *S. sonnei* phase I. and *S. sonnei* Re 4350 (■), *S. sonnei* phase I. and *S. sonnei* phase II. (▩) and *S. sonnei* phase II. and *S. sonnei* Re 4350 (□) were calculated.

1/100 dilution of 0.5% casein or 0.5% BSA was used for blocking, OD values increased (mean OD 0.993 for casein and 0.974 for BSA), but we were not satisfied with this result. Unfortunately these proteins may be bound to LPS non-specifically and can inhibit the binding of anti-LPS antibodies to epitopes of LPS and can diminish the sensitivity of ELISA tests. We tried to find more proper blocking agent. Normal goat serum was better than any dilution of casein or BSA. Sera from other animals were similar but not better than goat serum.

If we used goat serum after casein or BSA for re-blocking, the results were significantly better, which means the goat serum was capable of blocking non-specific binding sites better than casein or BSA. In the case when casein or BSA was used after goat serum for re-blocking, the results were worse, which left the goat serum LPS free. During casein or BSA re-blocking, these proteins could bind there. Our study demonstrates that ELISA using goat serum for blocking was the best, not only for detection of LPS and anti-LPS antibody reaction, but in cross reaction study too. This result was valid in general for all LPS types (S, R, and absolute R forms) and lipid A. This method was useful for estimation of differences between closely or distantly related antigens in one step without dilution series of sera. This new blocking technique was sensitive enough for detecting minor differences between antigens with high proportional coefficient of reproducibility.

ACKNOWLEDGEMENT

We gratefully acknowledge Dr. István Tóth of Veterinary Medical Research

Institute, Hungarian Academy of Sciences, Budapest for his helpful discussion and comments.

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