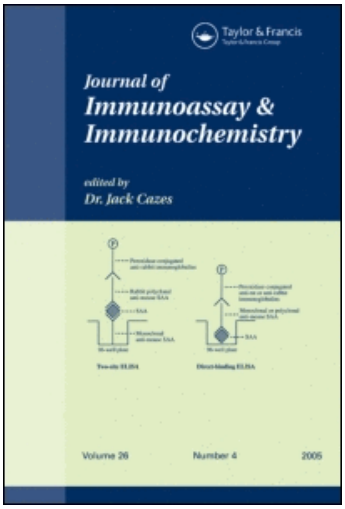

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INCREASED NON-SPECIFIC BINDING OF HEAT-TREATED PROTEINS TO PLASTIC SURFACES ANALYZED BY ELISA AND HPLC-FRACTIONATION

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ABSTRACT

Heat-treatment of proteins from human sera and bovine serum albumin caused an increase in their binding to microplates in a temperature-dependent manner. Quantitative data were obtained for ^{125}I-labelled human IgG, with binding of up to 400 ng of 56°-treated IgG per untreated well. However, only a minor increase in available antibody activity of adsorbed rabbit antibody was found upon pretreatment at 56°. The increased binding to microplates as a result of pre-incubation at raised temperatures was by high pressure liquid gel permeation chromatography demonstrated to be accompanied by polymerization. Inhibition of the direct binding of the proteins to plastic surface was achieved most efficiently by coating with non-interfering proteins followed by non-ionic detergent (Tween 20), which should also be present in the wash buffer.

INTRODUCTION

It is of crucial importance for the usefulness of enzyme linked immunosorbent assays (ELISA) to eliminate the non-specific binding to plastic surfaces after the initial binding of specific protein (antigen or antibody). To reduce non-specific binding in the second step the surface is saturated with non-ionic detergent and/or presumed non-interfering protein (1).

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Heat-treatment of sera (56°C for 30 minutes) has been advocated for measurement of antibodies in order to avoid the frequently observed inhibition of specific binding at low serum dilutions (2). We therefore analyzed the effect of heat-treatment of serum samples before application in ELISA for the estimation of human antibody. This treatment resulted in a significant increase in optical density in appropriate controls indicating non-specific binding of IgG. This prompted us to investigate more generally the adsorption of proteins to plastic surfaces as a function of preincubation at elevated temperatures. A report of preliminary findings has been published (3).

MATERIALS AND METHODS

Miscellaneous:

Tris buffered saline (TBS: 0.14 M NaCl, 10 mM tris/ HCl, 15 mM NaN_3, pH 7.4). TBS-Tween (TBS with 0.05% Tween 20). Diethanolamine 10%, 0.5 mM $MgCl_2$, pH 9.8 (DEA). Para-nitrophenyl phosphate (PNPP) (Sigma, Mo., USA), 1 mg/ml in DEA was employed as substrate for alkaline phosphatase (AP).

Sera and proteins:

Normal human sera (NHS) and normal bovine serum (NBS) were incubated at 20°, 37°, 45°, 56° or 65° for 30 minutes and diluted in TBS-Tween. Purified human IgG (Kabi, Stockholm, Sweden) was used in part of the quantitative binding studies. Small amounts

of IgG were iodinated by the Iodo-Gen method (4) to a specific activity of about 10 μ Ci/μ g, separated from free iodine on a Biogel P-10 column (100 x 20 mm) and added in a tracer dose.

Antisera:

IgG purified from rabbit antisera against human IgG, IgA and α_2-macroglobulin respectively were obtained from Dakopatts, Copenhagen, Denmark. AP-labelled affinity purified goat antibodies against human and rabbit IgG were obtained from Sigma, Mo, USA. Antibody against bovine serum albumin (BSA) was raised in rabbits, affinity purified (5) and subsequently labelled with ß-galactosidase (E. coli, E.C. 3.2.1.23, Boehringer Mannheim, West Germany) by means of the one-step glutaraldehyde method (6) using 300 μ g anti-BSA, 600 μg ß-galactosidase and 10 μ l 1% glutaraldehyde in a final volume of about 800 μ l. All antibody preparations were diluted in TBS-Tween.

Microplates:

Unmodified, flatbottomed 96-well polystyrene microplates (Nunc, Roskilde, Denmark), similar plates, especially treated for ELISA (Immunoplate II, Nunc, Roskilde, Denmark), and polyvinyl microtiter plates (Dynatech, Alexandria, Va., USA) were employed.

Quantitative studies with ^{125}I-IgG:

a. Direct binding of ^{125}I-IgG: Purified ^{125}I-human IgG was incubated at 20°C or 56°C for 30 minutes followed by dilution in TBS-Tween or 0.1 M bicarbonate buffer, pH 9.6. Samples of 200 μ l were incubated overnight in Immunoplates II. The plates were

either untreated, pretreated with TBS-Tween or pretreated with 0.1% HSA in TBS followed by TBS-Tween. After three times wash in TBS-Tween the single wells were cut out of the plate and counted in a gammacounter (Packard Auto-Gamma Scintillation Spectrometer). Ten percent trichloroacetic acid precipitated samples were included as totals, and the amount bound calculated.

b. Antibody binding of ^{125}I-IgG: Rabbit anti-human IgG antibody (Dakopatts) and normal rabbit IgG (NR IgG) at 20 mg/ml 0.15 M NaCl were treated at 56° or 20° for 30 min. In one experiment anti-human IgG antibody was diluted to 100 µg per ml bicarbonate buffer and further dilution (to 20 µg/ml, 4 µg/ml or 0) was done in bicarbonate buffer containing 100 µg NR IgG per ml to maintain a constant protein concentration. In another experiment the antibody preparation was diluted to 10 µg, 1 µg or 100 ng per ml of bicarbonate buffer without NR IgG added. In both experiments two hundred µl samples were incubated overnight at room temperature in the plate, which was then washed three times in TBS-Tween. Then was added 200 µl TBS-Tween, 1% normal rabbit serum, containing 250, 50, 10 and 2 ng human IgG, all including 2 ng ^{125}I-IgG. The wells were incubated overnight, washed and each well cut out of the plate and the radioactivity counted and calculations performed as above.

ELISA-Techniques:

a. Indirect methods: The wells of microplates were incubated with 300 µl TBS-Tween for 1 h before wash three times with TBS-Tween. Diluted test serum in 200 µl samples were applied to

the wells, which were incubated overnight at room temperature in a humidified chamber and washed trice. The adsorption of different proteins from serum were compared by adding anti-IgG, anti-IgA or anti-α_2-macroglobulin antibodies (Dakopatts) 1:1000 for 1 h, followed by wash and incubation with AP-goat anti-rabbit IgG antibody (Sigma) 1:1000. The wells were then washed and developed with substrate for 1 h at 37°C.

b. Direct methods: Microplates were either used untreated, pretreated with 300 µl TBS-Tween for 1 h before trice wash three times with TBS-Tween or pretreated with 0.1% human serum albumin (HSA) (Nordisk Insulin, Gentofte, Denmark) in TBS for 1 h before wash three times with TBS. Diluted testserum or -protein in 200 µl samples were applied to the wells, followed by incubation overnight at room temperature and wash three times. IgG adsorption was determined by addition of 200 µl AP-goat-anti-human IgG (Sigma) 1:5000 and incubation for 1 h at room temperature before wash and development.

Bound BSA was determined by addition to the wells of ß-galactosidase-labelled rabbit anti-BSA 1:5000, incubation for 18 h at room temperature, wash and subsequent development with o-nitro-ß-D-galactopyranoside (Sigma) at 1 mg/ml of 0.05 M phosphate, 1 mM $MgCl_2$, pH 8.2.

Colour development was read at 405 nm on a Titertek Multiscan spectrophotometer (Flow Labs, Irvine, U.K.). All samples were tested in triplicates.

High pressure liquid gel permeation chromatography-ELISA (HPLC-ELISA):

Serum samples, diluted 1:2, were centrifuged for 3 min. at 10^4 g in a Beckman Microfuge before the injection of 20 µl with a Water Intelligent Sample Processor (WISP™). The samples were by means of a Water 6000 A solvent delivery system pumped through a precolumn and the size separation column of 7.5 mm x 600 mm (TSK G 6000 PW, Toyo Soda) at a rate of 1 ml per min. The chromatography was performed in 100 mM Na_2SO_4, 20 mM phosphate, 7.5 mM NaN_3, pH 6.8. The separation characteristics were determined with the following purified markers: Helix pomatia (H.p.) (8900 K), poliovirus virions (P.V.) (6000 K), bovine thyroglobulin (Tg) (660 K), human IgG (150 K), bovine serum albumin (BSA) (67 K), ovalbumin (44 K) and cytochrome C (12 K). Fractions of 250 µl from the column were directly collected onto microplates by means of a microplate fraction collector (Skatron, Lier, Norway). The plates were prepared and processed for direct ELISA-methods for human IgG and for bovine serum albumin as described above. This HPLC-ELISA procedure has previously been described (7).

RESULTS

When human sera diluted 1:10 were preincubated at 56^o or 65^o a marked increase in the non-specific binding of serum proteins to the wells of TBS-Tween treated microtiter plates was observed. This was seen for a number of different proteins, including IgG, IgA and α_2-macroglobulin (fig. 1). Identical results were obtained with especially treated microplates (Immunoplates II) and with

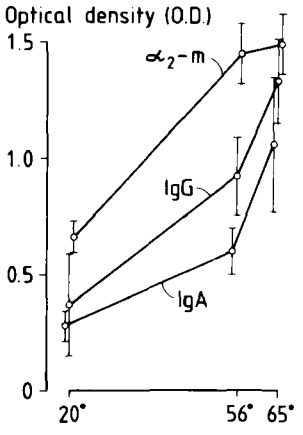

FIGURE 1. Binding of IgG, IgA and α_2-macroglobulin from NHS to TBS-Tween coated microtiter plates (Immunoplates II). NHS was preincubated at 20°, 56° and 65° for 30 min. and then diluted at 1:10 in TBS-Tween. Mean ± SD based on triplicate analysis of 4 serum samples.

unmodified polystyrene plates. Increased binding of IgG was present for 56°-treated NHS even at 1:1000 in TBS-Tween, and the amount bound corresponded to the amount bound from 100 ng purified IgG in bicarbonate buffer per well. The binding was markedly inhibited by incubation of the plates with 0.1% HSA in TBS prior to addition of TBS-Tween, and slightly further inhibited by 56°-treatment of 0.1% HSA before the incubation. Polyvinyl chloride plates showed greater variation in the duplicate determinations. However, increased binding of IgG from 56°-treated NHS was also observed, and this effect could be observed at a NHS-dilution of 1:5000. The increased binding was likewise inhibited by incubation of the plates with 0.1% HSA before addition of TBS-Tween (data not shown).

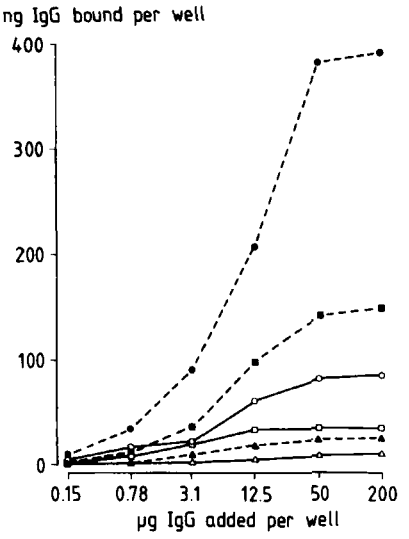

FIGURE 2. Quantitative estimation of IgG binding to microtitre wells. The wells were treated with TBS-Tween (■ or □), or 0.1% HSA in TBS (▲ or △), followed by wash three times in TBS-Tween, or non-coated (● or o). ^{125}I-IgG was added after incubation for 30' at 56° (solid signatures) or without incubation (open signatures). Note the difference in binding between heated and non-heated samples.

To allow a more quantitative estimation the binding of purified human IgG with ^{125}I-IgG added in a tracer dose was evaluated. Up to 400 ng of 56° treated IgG and only 80 ng untreated IgG was bound to untreated wells, while up to 150 ng 56° treated IgG could be bound to TBS-Tween coated wells (fig. 2). Abrogation of most of the binding was observed when the plates had been coated with 0.1% HSA before wash with TBS-Tween.

The functional activity of adsorbed 56°-treated compared to untreated rabbit anti-human IgG antibody was estimated. The amount of specific antibody used for coating was varied, while

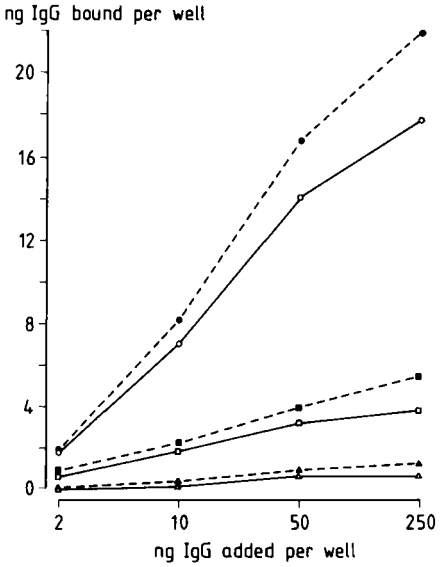

FIGURE 3. Binding of ^{125}I-human IgG (diluted in 10% normal rabbit serum) to polystyrene wells coated with 100 µg (● or o), 20 µg (■ or □) or 4 µg (▲ or △) rabbit anti-human IgG antibody per ml. 0.1 M bicarbonate buffer, pH 9.6. The protein concentration was kept constant by adding normal rabbit IgG. The antibody preparations were treated with 56° (solid signatures) or untreated (open signatures).

the total amount of immunoglobulin was kept constant (100 µg/ml). A modest increase in the binding of ^{125}I-IgG to the 56°-treated preparation was found (fig. 3). When the protein concentration was lowered a gradual increase in the percentage binding of the 56°C-treated compared to the untreated preparation was present, most significantly at 100 ng/ml.

The size distribution of the IgG which showed non-specific binding was investigated by HPLC-ELISA analysis of heat-treated NHS. Human serum samples were pre-incubated at 20°, 37° and 56°, processed for the separation as described in Materials & Methods

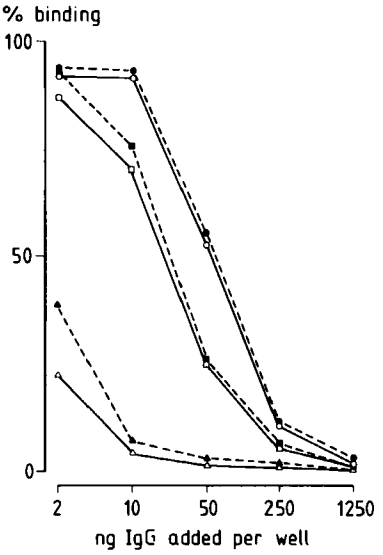

FIGURE 4. Percentage binding of ^{125}I-human IgG (diluted in 10% NRS) to polystyrene wells coated with 10 g (● or o), 1 µg (■ or□) or 100 ng (▲ or △) rabbit anti-human IgG antibody per ml 0.1 M bicarbonate buffer. In this experiment the immunoglobulin concentration of the antibody preparation was not kept constant by addition of NR IgG. The antibody preparations were treated with 56° (solid signatures) or untreated (open signatures).

and fractionated on a TSK G 6000 PW-column. IgG binding to the ELISA wells was greatly increased in the fractions eluted between V_o and V_{IgG} as a result of incubating the serum at 56°C. Serum treated at 37°C showed increased binding in the 150 K region only (fig. 5). Similarly, samples of NBS were heat-treated (37°, 45°, 56°) for 30 minutes, fractionated by the HPLC-column and delivered onto microplates. The HPLC-ELISA analysis showed greatly enhanced adsorption of BSA to TBS-Tween treated ELISA-wells between V_o and V_{BSA} and much less but still significant increase in the binding of BSA from 37° or 45°-treated NBS (fig. 6).

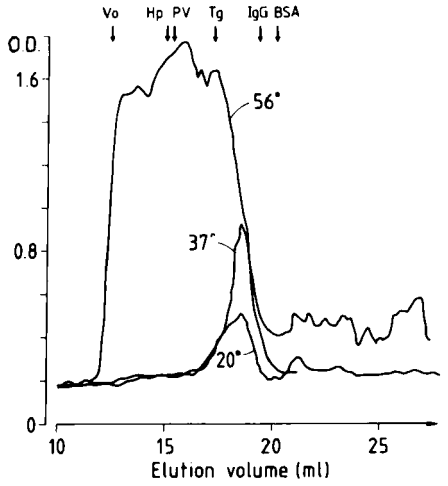

FIGURE 5. Estimation by HPLC-ELISA of the size distribution of IgG showing non-specific adsorption to polystyrene. Human serum was preincubated at 20°, 37° and 56° before fractionation. Fractions of 250 µl were collected onto TBS-Tween treated microplates and adsorption of IgG was estimated with AP-goat anti-human IgG. OD indicates substrate conversion at 405 nm. V_o denotes void volume. The elution volumes for the measured proteins are indicated (Hp. helix pomatia, P.V. polio virions, Tg bovine thyroglobulin, IgG is human IgG and BSA bovine serum albumin).

In HPLC-ELISA the enhanced binding of IgG from heat-treated NHS was substantially reduced when the wells had been preincubated for 1h in 0.1% HSA (fig. 7). HSA-treatment combined with addition to the wells of 25 µl 1% Tween 20 abrogated the binding of IgG in unheated or 37°-treated NHS, and substantially reduced the binding of IgG in 56°- treated serum.

DISCUSSION

In this report we have demonstrated up to 5 fold increased binding of proteins to plastic surfaces when serum or purified

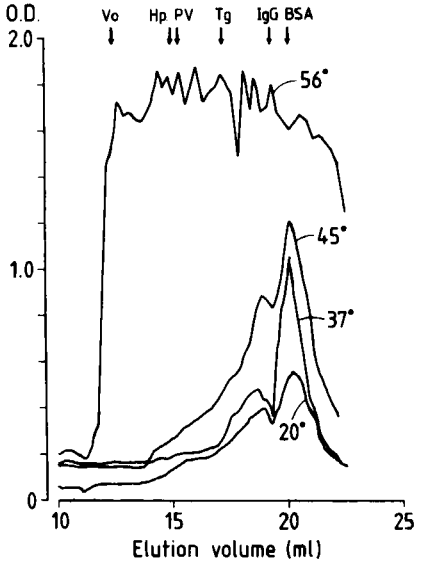

FIGURE 6. HPLC-ELISA for estimation of the BSA in NBS preincubated at 20°, 37°, 45° or 56°. Fractionation into TBS-Tween coated microplates, incubation overnight and addition of ß-galactosidase rabbit anti-BSA 1:5000. Subsequent development with substrate and reading of O.D. at 405 nm. Abbreviations as in fig. 5.

protein samples were preincubated at elevated temperatures. This effect was found for all 4 proteins examined and for polystyrene as well as polyvinyl surfaces.

Protein polymerization seems to be involved, as shown by HPLC-analysis of IgG and BSA. The nature of the bonds between the plastic surface and the proteins is not known, but heat-induced conformational changes as well as aggregation could possibly be involved in the observed increased binding.

By use of iodinated antigens it could be excluded that the increased response in ELISA was due to altered exposure of

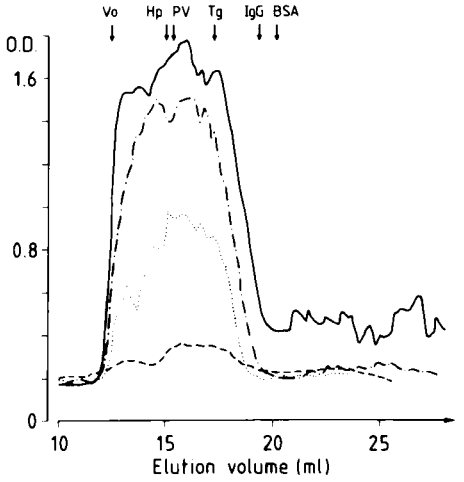

FIGURE 7. HPLC-ELISA for estimation of the IgG in NHS, pre-incubated at 56°. Fractionation into microtiter wells pre-treated with 0.05% TBS-Tween (——) or with 0.1% HSA in TBS (·—·). Alternatively, fractions were collected into wells to which had been added 25 ul 1% Tween in TBS after pretreatment either with 0.05% TBS-Tween (····) or with 0.1% HSA in TBS (– – –). Abbreviations as in fig. 5.

antigens on the solid phase after heat-treatment, rather than to increased binding of antigen to the surface.

Recently, Imagawa et al. (8) has shown increased background binding at 37° compared to 20° in ELISA for human chorionic gonadotropin. However, in that report they did not investigate the increased non-specific binding as a separate, pre-test phenomenon. Conradie et al. (9) found a rather modest (50%) increase in the ELISA readings, when IgG from sheep anti-HBsAg antibody was exposed to increased temperatures for 10 min. before coating and analysis of the antibody binding capacity. In agreement with this we found a modest enhancement in the binding of human IgG to solid phase rabbit anti-human IgG, when the antibody was incuba-

ted at 56° before coating the plastic (fig. 3). The enhancement was most pronounced at low protein concentrations (fig. 4). Thus it seems that the increase in protein adsorption is larger than the increase in activity of the bound antibody, possibly because of the heat-induced aggregation of immunoglobulins.

Other kinds of physico-chemical treatment of protein affect the characteristics of binding to solid phase. Increased activity of antibody coated to polystyrene beads has been shown after treatment of the antibody preparations with pH 2.5 (10, 11). This effect has been shown to coincide with partial denaturation of the protein, as demonstrated by circular dicroism spectroscopy (9).

In many procedures complement is inactivated by heat treatment (56° for 30 min.) and this procedure has also been suggested for ELISA measurement of antibodies at low serum dilutions (2). The observed increase in protein adsorption to plastic surfaces after heat treatment may limit the usefulness of this approach, if rigorous blocking is not performed, as e.g. with HSA and Tween.

As suggested (9, 11) the increased protein binding after heat-treatment to di rent plastic surfaces may prove advantageous when a high protein density is required. This would especially apply to situations where the desired antigen or antibody constitute only a minor proportion of the protein to be added in the coating procedure. However, our results with rabbit antibody only showed significant increase of solid phase antibody activity upon heat treatment, when coating was carried out at a

rather low protein concentration. This seems to limit the usefulness of this approach. Also neo-antigen determinants could possibly be created by protein denaturation.

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