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Short communication

The role of pH in modified ELISA procedures used for the estimation of functional antibody affinity

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Solid phase assays for the measurement of functional antibody affinity are increasingly being used in both clinical and research settings. The majority of such assays employ a chemical reagent to disturb antibody binding but relatively little is known about the properties of such reagents and the basis of their effect on antigen-antibody binding. We have evaluated the diethylamine (DEA) ELISA procedure for the measurement of functional antibody affinity in two independent assays, one for functional human IgG subclass affinity to an organism, *Moraxella catarrhalis*, and the other for measuring functional affinity of mouse monoclonals specific for the cat allergen *Fel d* I. DEA was shown to increase the pH of the buffering solution and it was this rise in pH that affected antibody binding. Alkaline buffer and DEA were equally efficient in the inhibition of binding of both the human IgG subclasses and the two mouse monoclonal antibodies to the solid phase. In contrast, pH was shown to have no role in the chaotropic effect of the ion, thiocyanate.

Key words: Functional affinity; ELISA procedure; pH effect; Thiocyanate; Diethylamine

Introduction

Increasing use is being made of modified antigen specific ELISA procedures for the estimation of affinity to large complex antigens (functional affinity or avidity (Karush, 1978)). These assays are based either on competitive inhibition of antibody binding to the solid phase by free antigen or the addition of reagents to the antibody dilution buffer or ELISA plate following antibody binding. Reagents used for this purpose have included

guanidine hydrochloride (Inouye et al., 1984), thiocyanate (Pullen et al., 1986), diethylamine (Hogben et al., 1986) and urea (Hedman et al., 1989). However, the properties of these reagents which inhibit antibody binding are not clearly understood.

In this study we have explored the role of pH in two modified ELISA procedures used for the evaluation of functional affinity. The functional affinity of naturally occurring human serum antibodies to a common micro-organism, *Moraxella catarrhalis* (*M. catarrhalis*) were determined by DEA and ammonium thiocyanate elution and the role of pH assessed in both techniques. Functional affinity characteristics of two murine mon-

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oclonal antibodies specific for the cat antigen *Fel d* I were also determined by DEA ELISA and the role of pH explored in this assay.

Materials and methods

Antigen preparations

The preparation of antigen for the *M. catarrhalis* ELISA has been described in detail elsewhere (Goldblatt et al., 1990). *Fel d* I (230 U/ml) was isolated from house dust by affinity purification using the monoclonal antibody Fd1a (Chapman et al., 1988).

Antibody preparations

Human serum pool. Serum standard: clotted blood from 60 apparently healthy adults was collected at a single sitting, then centrifuged and the serum pooled in equal amounts, aliquoted and stored at -70°C . This pool was referred to as the standard serum pool and used in each assay of functional affinity of human antibodies to *M. catarrhalis*.

Murine antibodies to *Fel d* I. The methods for generating the monoclonal antibodies 4F7 and 2D9 have been described in detail elsewhere (De Groot et al., 1988).

Miscellaneous reagents

Human sera, murine antibodies and thiocyanate were all diluted in 1% bovine serum albumin in phosphate buffered saline pH 7.0 with 0.05% Tween 20 (BSA PBS-T). All wash steps were performed using phosphate-buffered saline pH 7.0 with 0.05% Tween 20 (PBS-T).

General ELISA technique

Immulon no. 2 flat bottomed ELISA plates (Dynatech, Virginia, USA) were coated with *M. catarrhalis* whole cell preparation or *Fel d* I (100 ml, 1 mg/ml) in carbonate/bicarbonate buffer (pH 9.6) and maintained for 24 h at room temperature (or 48 h at 4°C) before the coating mixture was discarded and the plates allowed to air dry. Serial dilutions of human sera or monoclonal antibodies were made in duplicate in BSA PBS-T and added to the wells (80 ml/well). The plates were sealed to prevent evaporation and

incubated for 2 h at 37°C . The human sera/murine monoclonal antibodies were then discarded and the plate washed four times in wash buffer. Biotinylated antibodies (Zymed Laboratories (San Francisco, CA) IgG1 clone HP 6069, IgG2 clone HP6002, IgG3 clone HP6047, IgG4 clone HP6025 and total IgG clone HP6045) for the *M. catarrhalis* ELISA and a biotinylated goat anti-mouse antibody for the *Fel d* I ELISA were then diluted 1/1000 in BSA PBS-T. The wells were filled with 80 μl of the antibody solution and the plate incubated for a further 2 h at room temperature ($18-25^{\circ}\text{C}$). The plate was then washed four times in wash buffer following which 80 μl of a 1/50,000 solution of peroxidase conjugated streptavidin (Zymed Laboratories) were added. After standing at room temperature for 1 h the plates were washed four times in washing buffer before the addition of 80 μl per well of substrate solution containing 0.5 mg/ml of OPD in a solution of 0.05% H_2O_2 /0.1 M citric acid 0.1 M Na_2HPO_4 . The colour reaction was then stopped with 2 M H_2SO_4 (40 ml/well) following which the optical densities were measured at 490 nm using an ELISA plate reader (Titertek Multiskan, Flow).

DEA ELISA

In this assay DEA was used to inhibit antibody-antigen binding and was performed as described by Devey et al. (1988). Briefly, serial dilutions of human sera were made in BSA PBS-T in the presence or absence of various concentrations of DEA (0–40 mM). Optical density (OD) was plotted against serum dilutions and the leftward shift of the standard curve in the presence of DEA was measured at 50% of the maximum OD. Pre-incubation of ELISA plates with DEA was shown not to affect the binding of antigen to the solid phase (results not shown). To investigate the contribution of pH to the apparent chaotropic effect of DEA, assays were performed in the presence of diluent buffered to provide a range of differing pH values.

Thiocyanate ELISA

In these assays NH_4SCN was incorporated as the chaotropic ion as described by Macdonald et al. (1988). Briefly, the antibody incubation step

was performed as for the basic method except that a single dilution of antibody was used. Following a 2 h incubation with serum containing anti-*M. catarrhalis* antibody the plates were washed as before but before the detection antibody was introduced, BSA PBS-T NH_4SCN at different molarities (0–7.5 M) was added into the wells for 15 min at room temperature. The wells were then washed four times with PBS-T and then the ELISA was completed as described above. Pre-incubation of ELISA plates with NH_4SCN was shown not to interfere with antigen binding to the solid phase. Differing molarities of DEA and urea were used in the same way as NH_4SCN for comparison. Results were extrapolated from the ELISA by plotting the log of the % inhibition of the different molarities of NH_4SCN and determining the amount of NH_4SCN required to inhibit binding by 50%.

Results and discussion

To delineate the role of pH in the DEA-ELISA the pH of 1% BSA-PBS-T at different molarities of DEA was measured. 1% BSA PBS-T had a pH

of 7.2 and this rose to 10.8 for 20 mM DEA and 11.44 for 40 mM DEA. The pH of the DEA solution was stable over a period of 2 h and the pH gradient showed little inter-assay variability. Hence, the effect of increasing the molarity of DEA was to increase the pH. To investigate whether the effect of DEA on antigen antibody binding was pH dependent, in the *M. catarrhalis* system we neutralised the pH of 40 mM DEA with 1 M HCl and compared binding of standard serum at different dilutions in the absence of DEA, in 40 mM DEA and in neutralised 40 mM DEA-HCl. The results for IgG3 and total IgG are shown in Fig. 1. The affinity of IgG was lower than that of IgG3 due to the presence of IgG1 which ranked lower than IgG3. In contrast, the pH of 1% BSA PBS-T with increasing molar concentrations of NH_4SCN (0–7.5 M) showed a drop in pH (7.2–5.7). Neutralising the solution of NH_4SCN had no effect on the chaotropic effect of this ion (results not shown).

To investigate whether the effect of DEA PBS-T on antibody binding was totally pH dependent we compared, for IgG3 and total IgG, the leftward shift in the binding curve obtained using 30 mM DEA with that obtained using BSA PBS-T

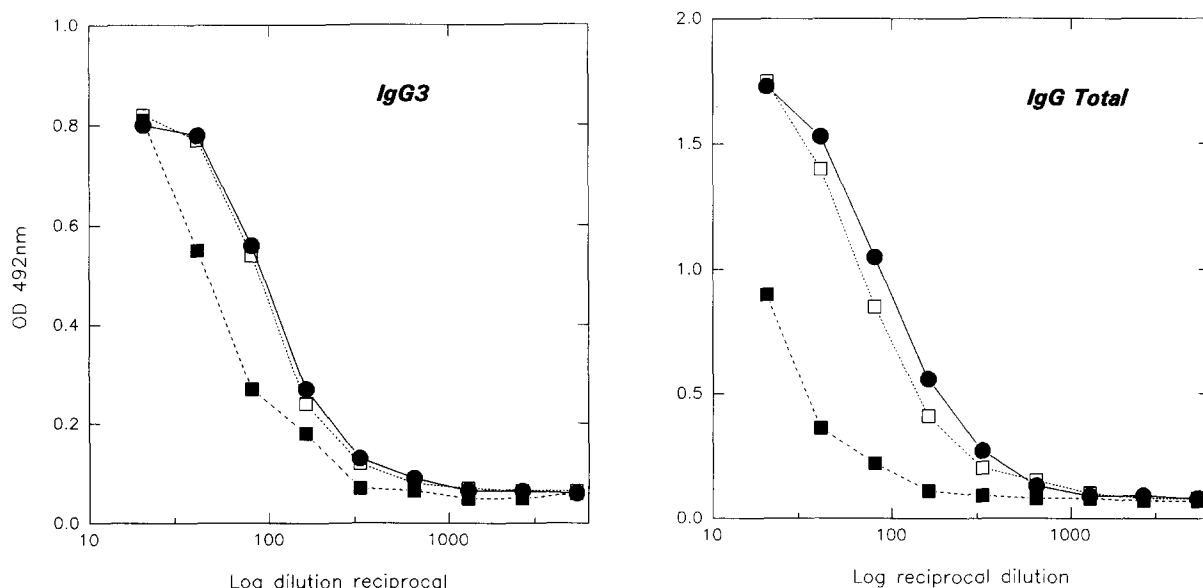


Fig. 1. Standard curves obtained for the binding of standard serum IgG3 and total IgG specific for *M. catarrhalis* in BSA PBS-T pH 7.2 (●), 40 mM DEA BSA PBS-T pH 11.44 (■) or neutralised 40 mM DEA BSA PBS-T pH 7.2 (□). Neutralising 40 mM DEA abolishes the effect of DEA on the binding of antibody to antigen.

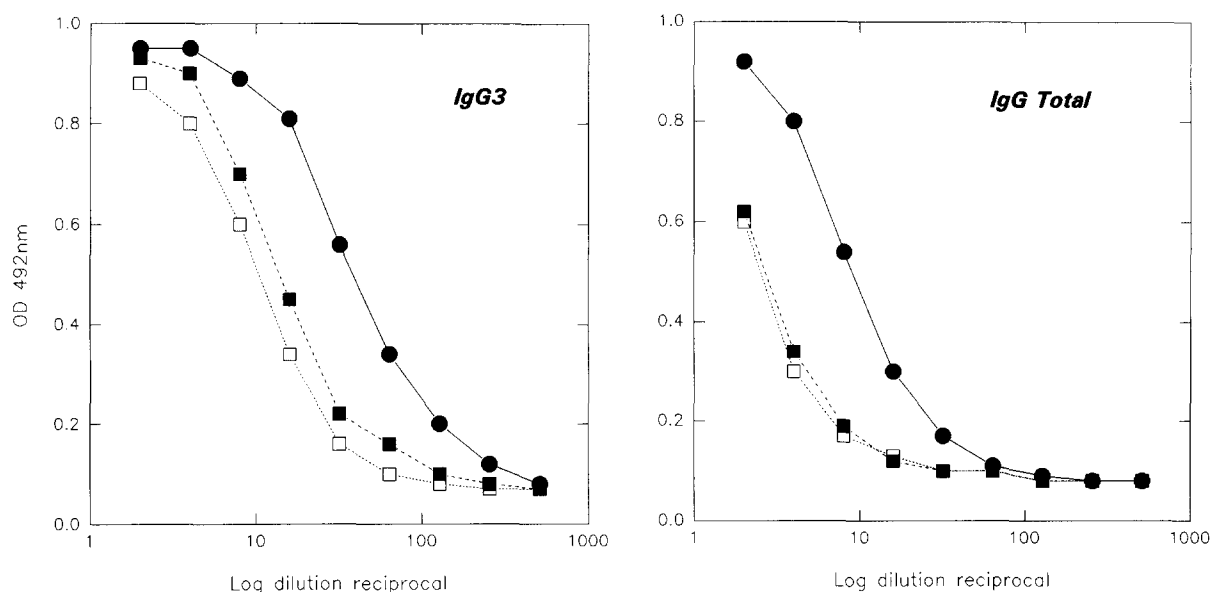


Fig. 2. Standard curves obtained for the binding of standard serum IgG3 and total IgG specific for *M. catarrhalis* in BSA PBS-T pH 7.2 (●), DEA BSA PBS-T pH 11.3 (■) and 30 mM DEA BSA PBS-T pH 11.3 (□). BSA PBS-T pH 11.3 is able to mimic the apparent 'chaotropic' effect of 30 mM DEA and produce a similar leftward shift in the binding curve. 30 and 40 mM DEA gave similar results.

NaOH pH 11.3 (see Fig. 2). The pH of the solution influenced antibody binding in the same way as DEA suggesting that the mechanism for

the DEA inhibition of antibody-antigen binding was essentially a pH effect.

The binding of the murine monoclonal antibodies 2D9 and 4F7 to a solid phase coated with *Fel d I* were compared in the presence of an increasing millimolar solution of DEA or 20 mM NaCl at increasing pH. The effect on antibody-antigen binding was again shown to be related to pH rather than any intrinsic properties of DEA and the results for 4F7 are shown in Fig. 3.

The effect of various chemical reagents on the binding of antibody to solid phase antigen in ELISA procedures has been recognised for some time but not fully understood. Our results suggest that the mechanism of action of a commonly used agent, DEA, is probably entirely due to the pH effect. Furthermore, the inhibition of antibody binding brought about by DEA could be reproduced with an alkaline buffer in both the systems studied. In contrast, increasing molar concentrations of the chaotropic ion, thiocyanate, reduced the pH of the buffer but neutralisation of the thiocyanate buffer had no effect on the reduction of antibody binding.

We suggest that millimolar concentrations of DEA are not chaotropic and that the effects of this agent on antigen-antibody binding can all be

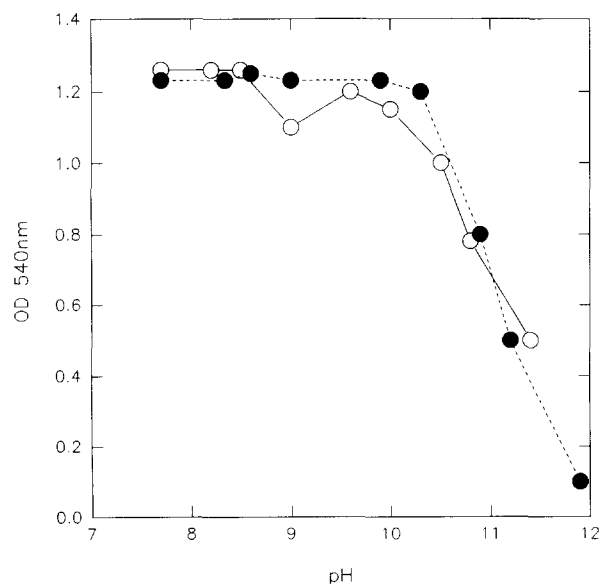


Fig. 3. Curves obtained for the binding of 29.3 ng of the murine monoclonal antibody 4F7 to a solid phase of *Fel d I* cat allergen in the presence of increasing molarities of DEA (○) or 20 mM NaCl-HCl (●) at increasing pH.

attributed to pH. Since pH changes may have differing effects on protein antigens depending on their charge and may chemically alter the antibody itself, the results obtained from such assays may not reflect true functional affinity. In contrast, Levison et al. (1970) concluded that, in general, chaotropic anions, such as thiocyanate, tend to unfold and dissociate macromolecules particularly through the breaking of hydrophobic bonds. For these reasons we believe that antigen inhibition or thiocyanate elution may be preferable solid phase methods for assessing functional affinity.

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