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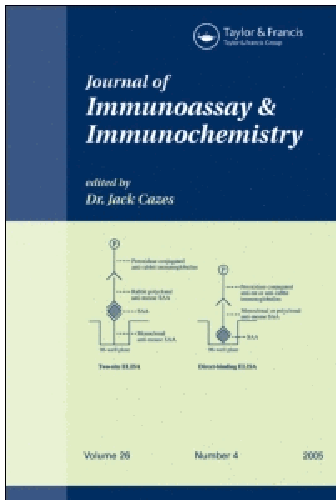

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Use of Solid-Phase C1Q to Remove Soluble Antigen/Antibody Complexes in an Inhibition Elisa for Streptococcal Cell Membrane Antigens

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USE OF SOLID-PHASE C1Q TO REMOVE SOLUBLE ANTIGEN/ANTIBODY COMPLEXES IN AN INHIBITION ELISA FOR STREPTOCOCCAL CELL MEMBRANE ANTIGENS

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Abstract

Solid-phase Clq was used to remove antigen/antibody complexes in an inhibition ELISA for low molecular weight streptococcal cell membrane (SCM) polypeptide antigens. To selectively fix IgM monoclonal antibody bound to antigen, binding was carried out in Clq-coated ELISA plates; transfer of supernatants to SCM-coated plates for ELISA permitted measurement of residual antibody. When inhibition occurred in the presence of Clq, the maximal binding was 72-98%. In the absence of Clq the maximum apparent binding was only 45-50%, which we attribute to displacement of the initially bound SCM antigen by solid phase SCM antigen. Removal of antigen/antibody complexes by solid-phase Clq during inhibition assays may facilitate analysis of low affinity antigen/antibody interactions. (KEY WORDS: anti-SCM antibody; ELISA; affinity; Clq binding; antigen/antibody complexes)

Introduction

A monoclonal antibody (mAb) raised against type 12 streptococcal cell membrane (SCM) and selected for cross-reactivity with glomerular basement membrane (1) was used to characterize various antigenic SCM polypeptides. Previous studies employing ELISA methods suggested that these SCM polypeptides comprised a

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repeating epitope and that these polypeptides had increasing epitope densities with increasing molecular size (2). To confirm this observation, inhibition experiments were designed. Antigenic SCM polypeptides (9.2, 7.0, 4.7, and 2.3 kd), a 13 kd SCM fraction eluted from an anti-SCM mAb column (2), and streptococcal type 12 M protein were incubated with the mAb at various dilutions under various conditions of time and temperature to examine the ability of these antigens to absorb antibody activity. Following absorption, the inhibitor/mAb mixture was tested for remaining antibody activity on SCM-coated ELISA plates. Inhibition of the mAb by these polypeptides in liquid phase was often inconsistent, and high levels of inhibition could not be achieved. Since the mAb was IgM, these results could be due to the low affinity of the mAb for low molecular weight SCM antigens in solution; a higher affinity for solid phase SCM antigen would then favor dissociation of the initial binding.

Since our goal was to compare the abilities of these different sized antigens to absorb antibody activity, we needed to achieve a wide range of inhibition so that the molar amounts needed for 50% inhibition could be compared. Since it has been reported (3,4) that solid phase Clq can be used to identify immune complexes, we explored the use of Clq to capture mAb bound to antigen. The results indicate that Clq can indeed be used in this way. Furthermore, the results confirmed our hypothesis regarding the relative epitope densities of the SCM polypeptides.

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Methods and Materials

Monoclonal antibody

Mice (Balb/c) were immunized with SCM as described (1). Spleen cells were fused with P3-Ag8-X63 non-secreting plasmacytomas and clones were selected for antibody activity with SCM and glomerular basement membrane as described (1). The mAb was isotyped with anti-mouse isotype mAb (Hyclone, Logan, UT) as described (2) and purified by euglobulin precipitation as described (2,5). IgM was quantitated by goat anti-mouse IgM mAb (Hyclone) as previously described (2).

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Antigens

Streptococcal antigens were derived from membranes prepared from type 12 *Streptococcus pyogenes*, group A, as described previously (6). Soluble antigen was prepared by trypsin digestion of SCM (100 mg enzyme:1 mg SCM) in PBS, pH 8.0, 20 hours at 37°. Protein content was determined for all antigen preparations by the bicinchononic acid (BCA) method (Pierce, Rockford, IL). Soluble SCM was passed over an anti-SCM mAb HPLC column (2) to prepare the affinity-purified antigen which was further purified and sized by HPLC-gel filtration (2). SCM tryptic digests were fractionated and polypeptides purified by HPLC-gel filtration (2). These SCM polypeptides were screened for reactivity with the mAb by ELISA as described (2). Streptococcal type-specific

M-12 protein was obtained from type 12 cells by hot-HCl extraction and purified as previously reported (7). SCM antigen-coated ELISA plates were prepared as described previously (2) by incubating the purified polypeptide antigens 4 ug/ml in carbonate buffer, 0.1 M, pH 9.6, in 96-well ELISA plates (Falcon) for 18 hr at rm temp. Uncoated plastic was blocked by incubating wells with bovine serum albumin (Sigma) 10 g/L in PBS for 3 hr at rm temp.

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Clq purification

Clq was prepared from fresh human serum by three rounds of precipitation at low ionic strength essentially as described (8). Purity was evaluated by cellulose acetate electrophoresis of the final preparation, the starting serum, and the supernatants. Clq was adjusted to 1 mg/ml in NaCl 0.75 mol/L, EDTA 10 mmol/L, acetate 20 mmol/L, pH 7.5. ELISA plates were coated with Clq as described (3). Clq was diluted in high-salt PBS (NaCl 0.64 mol/L, EDTA 10 mmol/L) to a concentration of 25 mg/L. Wells were coated with Clq 100 ul/well for 1 hour at room temp. The supernatants were removed and saved for further coatings. Uncoated sites were blocked by incubating the wells with bovine serum albumin (Sigma) 10 g/L in PBS, EDTA 10 mmol/L, 150 ul/well for 1 hour at rm temp. Plates were washed 5x with PBS and used immediately in inhibition assays.

ELISA and inhibition ELISA

To determine whether Clq-coated plates absorb free mAb, the mAb was incubated at various dilutions on Clq-coated plates. The well supernatants were transferred to SCM-sensitized plates and the remaining mAb activity was detected with horseradish peroxidase-labelled goat anti-mouse IgM antibody (Hyclone) as described below.

In the first set of experiments, the mAb was incubated with SCM antigens at concentrations of 0.005 to 10,000 ug/L in a variety of conditions: 1 hour at 37° followed by 20 hours at 4°; 20 hours at 4°; 1 hour at 37°. In the second set of experiments, similar incubations were performed in Clq-coated ELISA plates. The third set of experiments used BSA coated ELISA plates instead of Clq. The residual mAb in the well supernates was then transferred to SCM-coated ELISA plates for 1 hour either at rm temp or at 4°. Plates were washed 15x with PBS containing Tween 20 0.3 g/L. Horseradish peroxidase-conjugated goat anti-mouse IgM (Hyclone) 1:10,000 in PBS was added 100 ul/well for 1 hour at room temp. After washing the plates, the substrate, 2,2 azinodi-(3-ethylbenzthiozoline sulfonic acid) and hydrogen peroxide was added 150 ul/well and the plates were read at 10, 20, 30 minutes at 405 nm in a Dynatech (Alexandria, Virginia) Model 600 ELISA reader.

Results and Discussion

In the absence of Clq, residual mAb activity following incubation with the low molecular weight SCM antigens was incon-

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sistent. Inhibition of mAb activity never exceeded 50% even at SCM antigen concentrations in great excess of 10,000 nanograms; these results were obtained whether residual mAb activity was measured at 4° or at ambient temperature even though dissociation of mAb/antigen might be expected to be slowed at the lower temperature. It is possible that the mAb, being IgM, has a low affinity for the SCM antigens.

To determine whether Clq-coated wells were removing free mAb, the antibody was incubated on Clq plates at various dilutions and then transferred to SCM-coated plates. Comparison of the mAb incubated with Clq to mAb incubated without Clq indicated that Clq does not bind free mAb in this system (Figure 1).

The antibody was then incubated with the SCM antigens in the Clq-coated ELISA plates and individual well supernatants were transferred to SCM-coated plates to measure mAb activity. The results obtained following 1 hour inhibition at 37° in the presence of Clq and in the presence of BSA are compared in Figure 2. In the presence of Clq the maximum antibody binding achieved ranged from 72% to 98% for the various SCM antigens and remained constant at SCM antigen concentrations greater than 1000 nanograms (Table 1). In addition, Figure 2 shows that binding was specifically enhanced by the presence of Clq. The type 12 M protein served as a negative control (Table 1), since it is derived from the streptococcal cell wall and the mAb was raised against streptococcal cell membrane.

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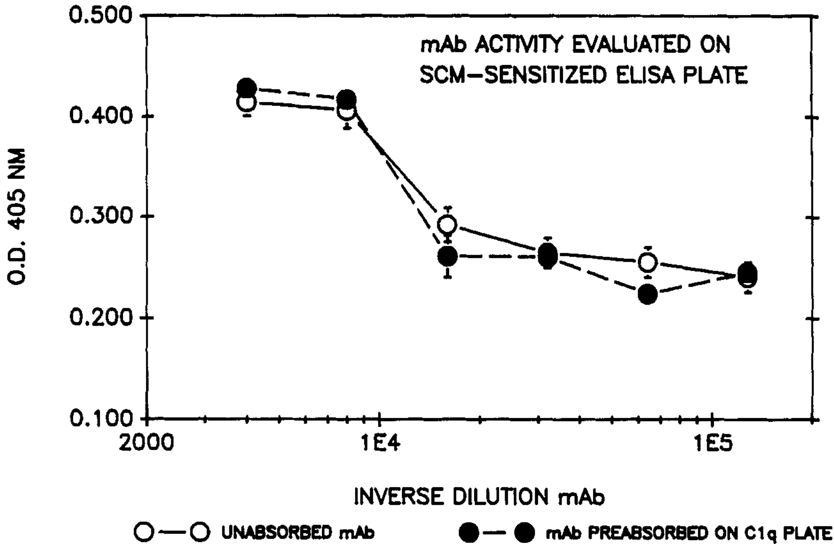

Figure 1. Clq-coated plates do not absorb mAb activity. Anti-SCM mAb was incubated on Clq-coated plates and then transferred to SCM-sensitized plates to assay mAb activity. The absorbed activity is compared to that of unabsorbed mAb. The points represent the means and standard errors of triplicate wells from two experiments.

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Table 1

MAXIMUM % INHIBITION ACHIEVED BY SCM ANTIGENS

INHIBITOR	WITHOUT Clq	Clq-ENHANCED
9.2 KD SCM	52 (50-54)	98 (90-100)
7.0 KD SCM	40 (38-45)	72 (71-90)
4.7 KD SCM	55 (52-57)	82 (80-90)
2.3 KD SCM	55 (50-62)	83 (74-90)
13 KD SCM ELUATE	54 (44-60)	83 (78-83)
M 12 protein	ND*	7 (0-10)

For Clq-enhanced inhibition, ELISA plates were coated with Clq and inhibition reactions were performed in the Clq-coated wells (1 hour, 37°). The supernatants from individual wells were transferred to SCM-coated plates and assayed for mAb activity. Inhibition performed without Clq was accomplished by incubating mAb with varying amounts of SCM antigens (1 hour, 37°). Reaction supernatants were transferred to SCM-coated plates and assayed for mAb activity. The average and range of per cent inhibition is given for each SCM antigen. * Not done.

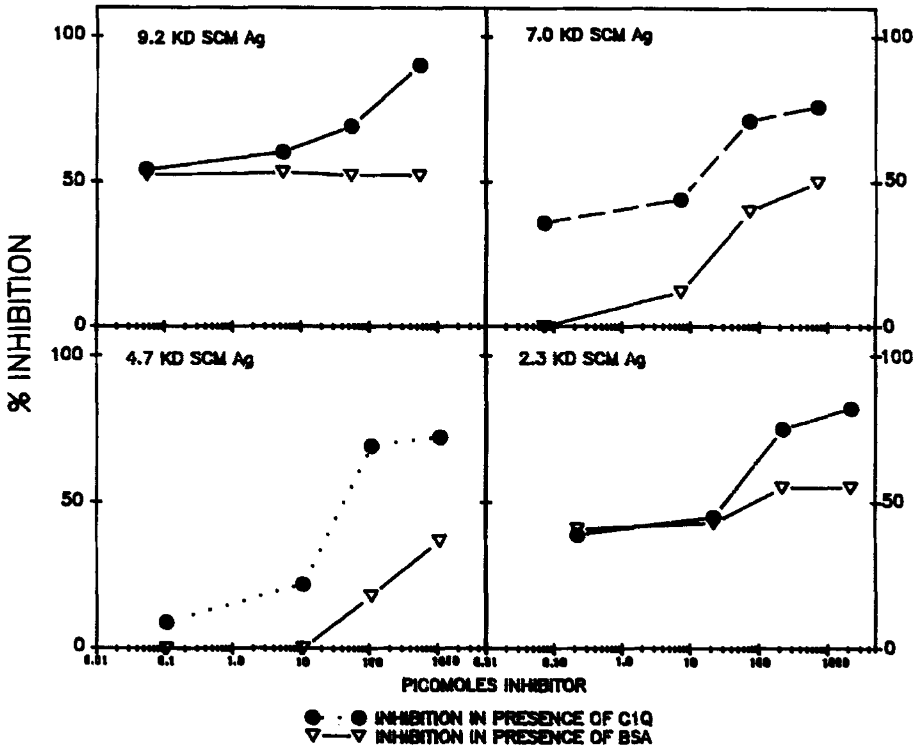

Figure 2. Solid-phase Clq specifically enhances inhibition of mAb in ELISA. Different sized SCM polypeptides were purified by HPLC-gel filtration and determined to be antigenic by ELISA. The polypeptides then were incubated as described (1 hr, 37°) with the mAb in ELISA plates coated with BSA or Clq. Individual well supernatants were transferred to SCM-coated plates to assay mAb activity. The points represent the means of three runs. Unabsorbed mAb gave O.D. 0.139-0.159. The O.D. for maximum inhibition using Clq ranged from 0-0.040; without Clq the O.D. ranged from 0.060-0.093.

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These results suggest that the immune complex-binding ability of Clq may be exploited to study the binding of antigens to low affinity mAb. If liquid phase antigens are easily displaced from antibody by solid phase antigens then their binding would be difficult to measure in ELISA without prior removal of immune complexes by solid phase Clq. Although this method was developed to address our particular need, we believe it may be useful in other systems where binding is difficult to measure due to a low-affinity antibody.

Finally, the results obtained with Clq-coated plates permitted a comparison of these different sized antigens on a molar basis. The amount of antigen required for inhibition appears to decrease as the size of the antigen increases. The pM required for 50% inhibition of mAb activity was 0.3, 10, 40, 110 for the 9.2, 7.0, 4.7, 2.3 kd SCM antigens, respectively. This is consistent with the results reported previously for these SCM antigens (2), and it suggests that the polypeptides are part of a larger protein antigen with a repeating epitope. This antigen may be the streptococcal membrane antigen cross-reactive with basement membranes first described by Markowitz and Lange (6) and may be responsible for inducing cross-reactive antibodies that lead to post-streptococcal glomerulonephritis.

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