

Variable Region Identical IgA and IgE to *Cryptococcus neoformans* Capsular Polysaccharide
Manifest Specificity Differences*

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*Running Title: IgE and IgA isotypes have differences in specificity

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Keywords: Immunoglobulin A (IgA), Immunoglobulin E (IgE), Antigen, nuclear magnetic resonance
(NMR), Isotype, Antibody

Background: Ig mu, gamma and alpha constant
regions can affect Ag specificity but this is
unknown for IgE.

Results: Variable region IgA and IgE differ in
specificity and cleavage rates compared to each
other and IgG subclasses.

Conclusion: Like IgG subclasses, the IgA and IgE
constant regions can affect antigen binding
specificities.

Significance: These results extend the principle
that the constant region can affect variable region
specificity to IgE.

**region-identical IgG subclasses and IgA,
consistent with an altered paratope. Both IgE
and IgA were opsonic for *C. neoformans* and
protected against infection in mice. In
summary, V region expression in the context of
the epsilon constant (C) region results in
specificity changes that are greater than
observed for comparable IgG subclasses. These
results raise the possibility that expression of
certain V regions in the context of alpha and
epsilon C regions affects their function and
contributes to the special properties of those
isotypes.**

ABSTRACT

In recent years several groups have
shown that isotype switching from IgM to IgG
to IgA can affect the affinity and specificity of
antibodies (Abs) sharing identical variable (V)
regions. However, whether the same applies to
IgE is unknown. In this study we compared the
fine specificity of V region identical IgE and
IgA to *Cryptococcus neoformans* capsular
polysaccharide and found that these differed in
specificity from each other. The IgE and IgA
paratopes were probed by nuclear magnetic
resonance spectroscopy (NMR) with ¹⁵N-
labeled peptide mimetics of cryptococcal
polysaccharide antigen (Ag). IgE was found to
cleave the peptide at a much faster rate than V

Following Porters' classic studies in the
1950s (1) immunoglobulins have been viewed as
having two functional domains: one for antigen
binding (2) and the second consisting of a
crystallizable (Fc) portion that interacts with Fc
receptors and complement. These domains were
viewed as functionally independent for over half a
century. However, this view began to unravel in
the 1990s when several observations were made
suggesting that the C region could influence Ag
specificity (reviewed in (3,4). In the past two
decades, studies from at least seven independent
laboratories have established that the C region can
influence the specificity and affinity of certain Abs
(5-12). This effect has been reported for IgM, IgG,

and IgA isotypes but IgE has not yet been explored.

C region-mediated effects in specificity and affinity have important implications for the generation of antibody diversity, primary and secondary B cell responses, and idiotype regulation (13,14). At least two mechanisms have been proposed for this effect: 1) C region effects on V region paratope structure (15) before and/or during antigen (Ag) binding and 2) subclass mediated differences in functional affinity leading to the recognition of new epitopes (6). The latter being a mechanism that may apply to IgG₃, which can self-aggregate through Fc-Fc receptor interactions to engage different epitopes through enhanced avidity (16). Although the molecular basis for these effects remains to be determined, a plausible mechanism involves C-mediated constraints on V region structure that affect the conformation of the Ig paratope. In this regard, circular dichroism studies showed that the C and V regions are structurally coupled and affect each other during Ag binding (17). In contrast, C region glycosylation does not contribute to this phenomenon (18).

A few groups have previously reported on differences in Ag binding affinities as well as paratope recognition between V-region identical IgA and IgG. Most recently, Tudor *et al.* revealed that switching a human IgG₁ to a monomeric IgA2 increased Ag specificity and binding affinity. They also found an increased function in anti-HIV-1 activity assays and altered epitope specificity (9). Earlier studies show that creating a chimera from a human IgA C region and mouse V region alters Ag specificity (7). A different study comparing a human monomeric IgA1 and its IgG₁ isotype revealed that though they bind the same epitope, these mAbs have significantly different binding affinities to the same Ag (8). Thus, like IgG isotypes there is evidence that the IgA C region affects its Ag specificity, regardless of avidity. However, there is no information as to whether the same principle applies to IgE.

Cryptococcus neoformans is a human pathogenic fungus that is remarkable for having a large polysaccharide capsule. The capsular polysaccharide glucuronoxylomannan (GXM) is an important determinant of virulence and a target for humoral immunity. The pathogenesis and outcome of human cryptococcal infection is

dependent on the interactions of both host and fungal derived factors (17). Initial infection by *C. neoformans* of an immunocompromised host occurs upon inhalation of spores into the lungs (19). Ab-mediated immunity can make a significant contribution to host defense and numerous protective mAbs have been reported. The efficacy of Ab-mediated immunity for *C. neoformans* was shown to be a function of Ab isotype, specificity, concentration, host immune function and host genetic background (reviewed in (18)). In general, the IgM and IgG subclasses except for murine IgG₃ have been shown to mediate protection against *C. neoformans* (19-22). In contrast, little is known about the role of IgA or IgE in protection against *C. neoformans*. A prior study reported that IgA mAb to GXM could function as an opsonin, though its IgG isotypes were more effective (20). However, there is no information on the potential role of IgE in its protection against *C. neoformans*.

The IgE isotype, which is found in low concentrations in serum, has four C region domains rather than the three all other Igs have (21). IgE plays the leading role in allergic responses by binding specific Fcε receptors (FcεR), found on a variety of immune cells, but mainly in basophils and mast cells with very high affinity. IgE-FcεR binding results in immediate hypersensitivity due to the release of immune mediators (21). The half-life of cell-bound IgE is in the order of weeks, and is responsible for the persistent sensitization of the cell to allergenic challenge. Interestingly, the additional C domain in the IgE structure replaces the IgG 'hinge' region, and has been shown to give the IgE molecule a more bent and less flexible shape. Upon binding its FcR, the IgE structure undergoes a significant conformational change in its second and third C region domains (C_{H2}, C_{H3}) (2). We have wondered whether this change may extend to its Ag-binding site and may thus affect Ag specificity.

In this study we evaluated the opsonic and protective efficacy of V region identical IgA and IgE to *C. neoformans* GXM derived from an IgG₃ mAb from the 3E5 family by spontaneous isotype switching. The 3E5 family of V region identical murine IgGs have been found to have proteolytic capacities impacted by their C regions. We then analyzed the IgE and IgA paratopes and abilities to

bind and cleave a small peptide Ag by NMR spectroscopy to ascertain whether they also demonstrated changes in specificity. Our results indicate that both IgA and IgE can protect against *C. neoformans*, both have higher rates of peptide cleavage than their IgG isotypes, and that the IgE C region can affect its specificity.

EXPERIMENTAL PROCEDURES

C. neoformans and Glucuronoxylomannan preparation. *C. neoformans* Strain 24067 (serotype D) was grown in Sabouraud media at 30°C. GXM was recovered from the same 24067 strain by shaking at 150 rpm at 30°C in Sabouraud dextrose broth, and isolation and purification with minor modifications (22).

Monoclonal antibodies. MAbs 3E5 IgA and IgE variants of mAb 3E5 IgG₃ were used in the form of hybridoma cell supernatant for ELISA experiments and as mouse ascites for survival experiments. The generation and preliminary characterization of the IgG, IgA and IgE variants of mAb 3E5 has been described (23). Briefly, murine IgE and IgA mAbs were purified by NH₄SO₄ precipitation followed by dialysis into 0.1 M Tris-HCl pH 7.4 and high-performance liquid chromatography on a Sephacryl-300 column (GE). The mAbs were then concentrated and mAb concentration was determined by OD₂₈₀ measurement.

Peptides. Peptide synthesis and its alanine variants were performed at the Proteomics Resource Center, Rockefeller University, New York, as described (15). For the NMR studies the P1 peptide was synthesized by Chem Pep to include ¹⁵N-labeled Methionine and ¹⁵N-labeled Leucine (SPNQHTPPW-[¹⁵N]M-[¹⁵N]L-K).

Binding and Competition ELISAs. Binding experiments to GXM was tested by ELISA using methods previously described (24,25). Briefly, 1 µg/ml of GXM in phosphate buffered saline (PBS) was used to coat polystyrene microtiter plates. The plate was then blocked with 1% bovine serum albumin in PBS. Primary antibody binding from mAb was detected using alkaline-phosphatase (AP) labeled goat anti-mouse antibody reagents (Southern Biotechnology, Birmingham, AL). Plates were developed with P-nitrophenol phosphatase (PNPP) substrate (Sigma, St. Louis, MO). All incubations were carried at 37°C for 1 h. and three washes were performed between

every step. Absorbance was measured in a microtiter plate reader at 405 nm.

Competition experiments were performed by inhibiting the binding of a mAb to GXM in the presence of a second mAb, as previously described (24). Briefly, ELISA plates were coated with 10 µg/ml of GXM, blocked and both mAbs were added; keeping one Ab (10 µg/ml) at constant concentration while changing the concentration of the second one starting at 100 µg/ml diluted serially by three. The binding of the Ab kept constant was detected by adding AP labeled goat anti-mouse isotype IgE (10 µg/ml) secondary Ab and developed with PNPP substrate (1 mg/ml). All incubations, washes and absorbance measurements were performed as mentioned above.

V_H and V_L sequence analysis. The V_L and V_H sequences of the 3E5 IgG subtypes were determined previously (26) and are available in Genbank (Accession numbers AY674874.1, AY674875.1, AY674876.1, AY674877.1, AY674872.1, AY674873.1, and AY679102.1). RNA was purified from hybridoma cells using Trizol (Life Technologies) according to the manufacturer's specifications. cDNA was generated using the Superscript III First-Strand synthesis (Life Technologies). Thus, we designed 3E5-specific primers based on the previously reported V gene sequences: 3E5VL-F, GATGTTGTGATGACCCAACTCCACT; 3E5VL-R, GATTTCAGCTTGGTGCCTCC; 3E5VH-F, ACGTGAAGCTCGTGGAGTCTGG; 3E5VH-R, TGAGGAGACTGTGAGAGTGGTGCCT.

Amplification was performed under the following conditions: 94°C for 1 min, followed by 30 cycles of 94°C 30s, 64°C 30s, 72°C 30s, with a final elongation step at 72°C for 10 min. PCR products were purified using the Qiaquick PCR purification kit (Qiagen) and submitted for standard sequence analysis at the Albert Einstein College of Medicine Genomics Core Facility. Sequences were edited using DNASTAR software and aligned using ClustalX.

Immunofluorescence (IF). Strain 24067 *C. neoformans* cells were washed with PBS and incubated with primary mAb (10 µg/ml). Then, cells were washed with blocking solution (1% Bovine serum) and incubated with secondary fluorescein-labeled goat anti-mouse (GAM)

isotype specific Ab (10 μ g/ml). After this incubation, cells were washed and resuspended in 0.1 M of n-propyl gallate. A few microliters of the suspension were fixed into a glass slide and its immunofluorescence pattern was visualized with a microscope. All incubations were carried out at 37°C for 30 min and cells were washed three times between each incubation.

Phagocytosis assay. To study phagocytosis, macrophage-like J774.16 cells and *C. neoformans* strain 24067 cells were used employing assays that were previously described (27). Briefly, 3×10^4 J774.16 cells were activated with 50 U/ml IFN- γ and 1 μ g/ml of LPS in DME medium and then incubated overnight at 37°C in a 10% CO₂ incubator. The following day, fresh medium containing *C. neoformans* cells for a final ratio of 5:1 to macrophages was added and incubated for 2 h in the presence or absence of specific mAbs at 37°C. Cells were then washed with PBS, fixed with cold methanol and stained with Giemsa. Phagocytosis experiments were done at least three times in triplicate, 100 macrophages with ingested or non-ingested crypto were counted per well and result reported as percent phagocytosis.

Survival studies. Six to eight week old BALB/c mice were used in survival experiments. 100 μ g of either 3E5 IgA or IgE mAb were injected intraperitoneally (IP) 2 hrs before infecting with 1×10^6 *Cryptococcus neoformans* cells intravenously via tail vein. Survival was monitored daily.

NMR Spectroscopy. IgE was concentrated to 32 μ M in 0.1 M Bis-Tris and 0.15 M NaCl pH 6.5 buffer. 100 μ M P1 (Chem Pep) was added just before NMR analysis for the 37°C experiments. ¹⁵N-¹H^N heteronuclear single quantum coherence (HSQC) spectra (28,29) were recorded using ¹⁵N labeled P1; P1 alone, and IgE/P1 complexes on a Bruker Avance spectrometer at 600 MHz capable of applying pulse field gradients along the z-axis. Studies at 37°C were done immediately upon incubation of P1 with the mAb, as a series of one-dimensional 11.5 min HSQC runs, spanning 3 h. Experiments were processed using NMRPipe. Analysis was done using either NMRPipe (30) or NMRViewJ (31).

IgE/IgA Modeling. Two modeling approaches were used to investigate how the constant regions could affect V region structure. In the first approach, molecular models for IgA and IgE Fab

domains were made using the Antibody Modeler module of MOE2013.08.02 (32). For each molecule a total of 25 intermediate models were generated; 5 for backbone and 5 side-chain models per backbone model. These intermediate models were optimized by energy minimizations to a root mean square (RMS) gradient of 0.01 kcal/mol/Å² using AMBER10 Force-field (33) with reaction field implicit solvation model; the external and internal dielectric constants were 80 and 1 respectively. 8 and 10 Å were used as cut-off distances for non-bonded interactions. These 25 intermediate models were ranked according to their GB/VI scores (34). The highest ranking models were selected for both IgA and IgE. These models were then optimized further with AMBER10 Force-field to a RMS gradient of 0.0001 kcal/mol/Å². In the second approach, a structural model of the 3E5 IgA/IgE variable region (Fv) was generated from the corresponding amino-acid sequence using the Prediction of Immunoglobulin Structure (PIGS) server with default settings (35). The model was generated using the similar anti-GXM immunoglobulin 2H1 IgG₁ structure (PDB ID: 2H1P) as a template (36). The position of conserved amino acid side chains was maintained, while the non-conserved amino acid side chains were modeled with SCWRL4 (37). The model was then optimally superimposed with the corresponding portion of the 3E5 IgG₃ Fab crystal structure (PDB ID: 4HDI) (38) in PyMOL (39) and amino acid substitutions were highlighted.

RESULTS

Variable gene sequences of 3E5 IgE and IgA. The 3E5 IgE and IgA mAbs are spontaneously arising switch variants derived from the 3E5 IgG₃ hybridoma by the sib selection technique (23). To ensure the fidelity of the switching process with regards to V region sequence, the V_H and V_L of the 3E5 IgE and IgA were sequenced. The V_L sequences of the IgA and IgE variants were found to be identical to all of the IgG subtypes. However, for the V_H sequences we found two polymorphisms distinguishing the IgA and IgE variants from the IgG variants (Figure 1). These polymorphisms presumably arose during the isotype switching process and result in two amino acid differences in the V_H; position 65 Lys→Arg, located in a buried region between CDR2 and

CDR3, and position 97 Ala→Val, located in the CDR3 region in IgE and IgA variants (Genbank accession numbers KM872102-KM872105). Hence, the IgA and IgE variable regions were 100% identical to each other, but they differed in two amino acid positions from the IgG variants. Furthermore, SDS-PAGE showed that heavy and light chains were the expected mass for both IgE and IgA (data not shown).

Reactivity of 3E5 IgE and IgA with GXM and C. neoformans. Both 3E5 IgE and IgA bound to GXM by ELISA (Figure 2A) and competed with one another (Figure 2B), as would be expected for two Abs with identical V region usage. Binding to *C. neoformans* was studied by indirect IF. Both IgE and IgA bound to the capsule of *C. neoformans* in an annular pattern that demonstrated some subtle differences in the intensity and localization of immunofluorescence. IgE bound more diffusely, while IgA had a more clustering or punctuate binding pattern (Figure 3). These subtle differences are what lead us to believe that there may be isotype-related differences in Ag binding in the first place. Similar differences have been found with V region identical mouse-human chimeric (8) and murine IgG subclass (40) families that share identical V region usage.

Reactivity of 3E5 IgE and IgA to peptide mimotopes. To explore whether 3E5 IgE and IgA differed in specificity from other 3E5 IgG switch variants we tested their reactivity with a peptide mimotope (P1) that had been modified by sequential replacement of alanine at all amino acid positions. The reactivity of 3E5 IgE for the alanine-replaced peptides was remarkably robust relative to that of 3E5 IgA or the IgG subclasses 3E5 IgG₁ and 3E5 IgG₃ (Figure 4A). Furthermore, when we introduced replacement substitutions to the peptide involving other amino acids there was relatively little loss of reactivity for 3E5 IgE compared to the other isotypes (Figure 4B). These results indicate that the reactivity pattern of IgE was very different from that of IgA, which in turn was remarkably similar to that of IgG1.

NMR Spectroscopy with [¹⁵N]M10-[¹⁵N]L11-labeled P1. To explore the paratopes of the 3E5 IgE and IgA mAbs, we studied their in-solution binding to the P1 peptide with two ¹⁵N-labeled amino acids by heteronuclear signal quantum coherence (HSQC) and mapped chemical shift

perturbations. We measured the ¹⁵N and ¹H^N HSQC correlations of P1 when bound to the mAbs at both 25°C and 37°C, and compared the spectra to that of P1 without Ig as well as to the spectra of a control mAb, MOPC195 (murine IgG_{2b}), incubated with P1. We expected two new NMR signals upon IgE and IgA binding to P1, chemical shift perturbations upon mAb binding, corresponding to changes in the chemical environment of the ¹⁵N-labeled amino acids. We found that both mAbs bound P1 at 25°C and 37°C, as evident by the significant decrease in the intensities of the resonance peaks resulting from enhanced relaxation effects due to Ab binding, where each peak represents one of the ¹⁵N labeled amide bond on either M10 or L11.

The proteolytic activity of both IgE and IgA was investigated by analyzing NMR peak intensities of labeled amino acid M10 within the peptide P1. Within a 3-5 h incubation period of IgE or IgA and P1 at 37°C, we saw the appearance of new resonance peaks (Figure 5). The disappearance of bound P1 resonances and appearance of new resonance peaks were time dependent, suggesting that the peptide was being modified and possibly cleaved by both IgE and IgA. Yet the proteolytic activity differed between IgE and IgA. The intensity changes fit well into an exponential function for IgA, but IgE intensities fit into a sigmoidal function indicating a more complex proteolysis mechanism (Figure 6). The cleaved L11 peak is not visible in IgE complex suggesting M10-L11 peptide bond is targeted by this mAb. In the peptide:IgA complex, extra peaks were visible, at least one fragment still possessed the [¹⁵N]L11 residue, indicating the M10-L11 peptide bond was still intact (Figure 5). The fits and rates are summarized in Table 1. The HSQC spectra of P1 alone were also collected at 37°C, resonance peaks did not change and there were no visible new peaks, suggesting that P1 is stable in the buffer used (data not shown). Mass spectrometric analysis of the IgE+P1 solution after NMR analysis at 37°C revealed the appearance of two fragments with the mass (m/z) of ~1196 (SPNQHTPPWM/LK) and ~1012 (SP/NQHTPPWM/LK) confirming proteolysis of the peptide (Figure 7A). Mass spectrometric analysis of the IgA+P1 solution after NMR analysis at 37°C revealed the appearance of two fragments with the mass (m/z) of ~1126

(SP/NQHTPPWML/K) and ~1012 (SP/NQHTPPWML/LK) (Figure 7B). As a positive control, we have seen the same phenomenon, with similar if not identical P1 fragmentation with the 3E5 family of IgG isotypes (15). As a negative control we used murine MOPC195+P1 and did an identical NMR time-course where the HSQC spectra of [¹⁵N]M10-[¹⁵N]L11-labeled P1 did not show changes in P1 peak intensities or positions upon MOPC195 mAb addition (data not shown).

Opsonization and protective efficacy of 3E5 IgE and IgA for C. neoformans. We evaluated the opsonic efficacy of 3E5 IgE and IgA for *C. neoformans* with J774.16 cells. In designing these experiments we needed to account for the fact that antibody binding to the capsule of *C. neoformans* can mediate changes that allow for phagocytosis through the complement receptor (CR) in the absence of complement (41). This phenomenon appears to result from the direct interaction of capsular polysaccharide with the CR and can be prevented by co-incubation with CR blocking antibodies to CD18 and CD11b. The phagocytosis of 3E5 IgE was partially blocked with both antibodies to FcR and CR while blocking CR completely abrogated the opsonic capacity of IgA (Figure 8A). Administration of either 3E5 IgE or IgA to mice before infection with *C. neoformans* prolonged survival (Figure 8B), as has been shown for other IgG to GXM (26).

IgE/IgA Modeling. Using the Antibody Modeler approach to elucidate the conformational differences resulting from differences in constant regions, the Fab structural models of 3E5 IgE and IgA were superposed with the crystal structure of 3E5 IgG₃-Fab (PDB:4HDI)(15) on either their light chains (Figure 9A and 9B, RMSD of 0.873 Å) or their heavy chains (Figure 9C and 9D, RMSD of 0.678 Å). The constant regions influence the framework conformations and both the light chain and heavy chain CDRs (Figure 9 graphs). When the three molecules were compared, the IgA-IgE pair shows smaller RMSD deviations for both framework and CDR residues than the IgG₃-IgA and IgG₃-IgE pairs. This indicates a higher structural similarity between IgA and IgE molecules, with IgG₃ being the most structurally distant. These results may explain the differences seen in catalytic rates; the C regions may affect CDR conformations within the V regions. IgA and IgE cleave the same peptide

much faster than IgG₃, indicating a similar proteolytic mechanism manifested by their similar structures. This model was confirmed by the other approach using the PIGS server with default settings (35). Superposition of the IgE/IgA Fv model with the IgG₃ Fv region yielded an RMSD of only 0.679 Å following alignment of 1553 atoms (data not shown). This indicates a high similarity between the two and corroborates the previous model.

DISCUSSION

In this study we have explored the effect of alpha and epsilon C regions on the fine specificity of antibodies to *C. neoformans* and their ability to opsonize and protect against this fungus in macrophages and mice, respectively. There is now conclusive evidence from several laboratories that expressing identical V regions in the context of IgM, IgG, and IgA can alter Ab specificity and affinity but no comparable information is available for IgE. Here we investigate whether the epsilon C region also had the capacity to affect V region structure resulting in specificity and affinity changes.

We sought to compare IgE and IgA variants derived by isotype switching from an IgG₃ hybridoma (3E5), that had already provided us with a set of V region IgG subclasses. However, the immunoglobulin gene sequences of the 3E5 IgA and IgE clones were found to have changes in the VH at positions 65 and 97. Position 97 is in the CDR3, therefore a surface residue, and corresponds to a replacement of valine in the IgG variants for alanine in both IgA and IgE. Alanine and valine are small hydrophobic amino acids that in theory may not mediate much change in protein structure. Position 65 is also a surface residue based on the 3E5 IgG₃ crystal structure, and although Lys→Arg is a charge-conserving change, Arg has a significantly larger R group. These differences could potentially lead to small changes in secondary and tertiary structure between these Igs and the IgG family. However, we created an *in silico*-generated model of the IgE/A Fab incorporating the amino acid substitutions and superimposed it onto the 3E5 IgG₃ model (Figure 9). The resulting RMSD of only 0.678 Å indicates that the changes due to these mutations are at most, minimal. Most importantly, the IgA and IgE Abs have identical V regions and since V region

identical IgA and IgG have already been shown to differ, the comparison between the 3E5 IgA and IgE pair allows us to extrapolate information to the epsilon C region. Consequently this Ab set provides us with the opportunity to explore whether the epsilon C region, like the gamma and alpha C regions, can affect Ab specificity.

In early studies we noted that V region identical Abs differing in C region produced subtle differences in IF pattern when bound to the *C. neoformans* capsule (8,40). In fact, it was differences in IF pattern that provided the early hints that led to our observations that the C region affected V region binding (8). Consequently we carried out similar studies for IgA and IgE and again observed similar subtle differences in their IF pattern for IgA and IgE. Although this is suggestive of differences in fine specificity, small differences in hinge angle and flexibility could translate into avidity differences that could also be responsible for different binding patterns. Nevertheless, the finding of subtle differences was consistent with the possibility of isotype-related differences in specificity. Since the carbohydrate epitope recognized by these Abs in GXM is not known and there are no oligosaccharides available to probe single Fab interactions with Ag, we have used peptide mimetics to explore differences in specificity in solid support binding by ELISA and in solution binding by NMR. Furthermore, we have made alanine substitutions as well as conserved and non-conserved changes in the peptide mimetic for fine dissection of specificity. IgE binding to the mutated peptide mimotope was very different from that of the IgG subclasses and IgA, with the major finding being that there was little perturbation of its binding capacity despite replacement of amino acids with non-conserved residues. This observation was also consistent with C region mediated changes in specificity and suggested that the V region binding pocket, when attached to the epsilon constant region, was less influenced by its local environment than by its global conformation, such that its conformation was not easily perturbed. In contrast, the binding pattern of IgA was similar to that observed for V region identical IgG subclasses (15).

Previously we have used solution NMR studies of the interactions between V region identical IgG subclasses and the peptide [¹⁵N]M10-[¹⁵N]L11-labeled P1 to obtain direct

evidence for C region mediated changes in paratope and the same experimental approach was used with the 3E5 IgA and 3E5 IgE Abs. Similar results were independently reported by another group who found that different C regions affected the catalytic efficiency of V region identical Abs differing in isotype (42). As was described for the 3E5 IgG family, both 3E5 IgA and IgE cleaved the peptide as evident by the appearance of new resonance peaks and MS data of cleaved fragments. However, IgE was very different from the other 3E5 isotypes in manifesting very rapid cleavage kinetics, although the three distinct fragments seen in NMR and the two seen by MS match the same size and positions as the 3E5 IgG results (15). This indicates a similar cleavage mechanism as the IgG isotypes. The sigmoidal behavior of proteolysis by IgE can be explained by coupling to an allosteric mechanism; P1 binding induces conformational rearrangements within the CDR resulting in proteolytic capability. Probing this allosteric mechanism and conformational rearrangements will be the subject of future studies. On the other hand, the intensities fit into an exponential decay function for IgA. Proteolysis of P1 by IgA resulted in fragments that differed in size from the IgG and IgE. Thus indicating that IgA proteolytic activity was unique as there was an additional peptide bond targeted by IgA: L11-K12, differing from the other isotypes.

Both 3E5 IgA and IgE promoted phagocytosis of *C. neoformans* by the murine macrophage-like J774.16 cells. However, there were differences in the mechanism of opsonization. Blocking of complement receptor with Abs to CD18 and CD11b completely abrogated phagocytosis by IgA, but not IgE. Since Abs to *C. neoformans* have been shown to mediate opsonization of yeast cells by a unique mechanism whereas Ab binding to the capsule promotes an interaction between the polysaccharide capsule and the complement receptor independent of complement we conclude that IgA is promoting phagocytosis through that mechanism. In contrast, blocking of CD18 and CD11b caused only a partial reduction in phagocytosis by J774.16 cells whereas blocking of the FcγR produced a much greater inhibition of 3E5 IgE-mediated phagocytosis. Although IgE interacts primarily with the FcεR there is evidence that it can also interact with the FcγR (43). Furthermore, IgE and

IgG immune complexes have been shown to trigger similar reaction from macrophages (44). Passive administration of 3E5 IgA and IgE to mice prior to *C. neoformans* infection resulted in prolonged survival extending a potential role in protection against fungal infection to these isotypes.

In summary, our results extend the concept that isotype can affect V region specificity to the epsilon constant region and show the potential of this isotype to mediate opsonization of and protection against *C. neoformans*. In fact, the effects of IgE on fine specificity as measured by peptide mimetic binding and NMR were much greater than observed for the IgG subclasses or IgA suggesting that this constant region affects the paratope to a greater extent than the other C

regions. Whether such C region effects contribute to the propensity of this isotype to be elicited by certain allergens is unknown. Although IgE responses are generally considered deleterious in the context of promoting immediate hypersensitivity, we note that there is recent interest in the use of this Ab class in cancer therapy (43). Most IgE is thought to be sequestered in tissue by its strong interaction with FcεR but our observations imply a potential role in defense against fungi in addition to its classical role in protection against helminths. Our findings add to the growing body of knowledge of C region effects on binding and protection and suggest the need for similar studies in other systems.

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FOOTNOTES

*This work was supported by NIH awards HL059842, AI033774, AI052733, AI033142. AJ wishes to acknowledge support from the Institutional AIDS training grant T32-AI007501 and the MSTP training grant, T32-GM007288. AB wishes to acknowledge NIH awards T32-GM007288 and R37-AI033142.

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FIGURE LEGENDS

FIGURE 1. 3E5 family Ig variable region sequence alignments. (A) Variable light chain comparison. (B) Variable heavy chain comparison. 3E5 IgG sequences are identical and combined here as “IgG”; the two polymorphisms are highlighted.

FIGURE 2. A. Binding of changing concentrations of mAbs 3E5 IgA and 3E5 IgE to immobilized GXM. Isotype specific secondary abs were used as well as anti-kappa secondary Ab. Inset diagram represent the schematic for the ELISA configuration. B. Competition ELISA of mAb 3E5 IgE with changing concentrations of mAb 3E5 IgA to immobilized GXM. Secondary Ab used is GAM-AP IgE. The schematic represent the ELISA configuration.

FIGURE 3. Immunofluorescence of strain 24067 stained with mAbs 3E5 IgA and 3E5 IgE.

FIGURE 4. A. Alanine substitution of peptide P1 Ag mimetic binding profiles. B. Conserved substitution binding profiles.

FIGURE 5. IgA and IgE 2D NMR spectra at 37°C after a 3-5 h incubation period. Three new peaks have the same locations in both spectra, while the IgA spectrum shows the appearance of three additional peaks.

FIGURE 6. Proteolysis of peptide P1 by IgE and IgA at 37°C. A. The proteolytic activity of IgE, and B. IgA, was analyzed by fitting observed NMR intensities of [¹⁵N]Met-10 and [¹⁵N]Leu-11. IgE and IgA show different catalytic mechanisms evidenced by different intensity fits. Intensities arising from IgE catalysis fit well into a Boltzman Sigmoid suggesting an allosteric mechanism regulating P1 proteolysis whereas intensities from IgA catalysis fit to an exponential function indicating a more simple proteolytic mechanism. M10 and L11 indicate Met-10 and Leu-11 of the free peptide while M10' and M10'' indicate Met10 from cleaved P1 fragments.

FIGURE 7. IgA and IgE 25°C Mass Spectrometry results. (A) IgA+P1 fragmentation, the appearance of two new peaks at 1012 m/z and 1126 m/z is seen after 3 h, (B) IgE+P1 fragmentation, two new peaks at 1012 m/z and 1196 m/z are seen after 3 h.

FIGURE 8. A. J774 cells and *C. neoformans* were used to study the phagocytosis efficacy of the mAbs. B. Survival of Balb/c mice with either NSO or the two variants of 3E5 mAb. The abs were administered two h prior to infection.

FIGURE 9. Comparison of IgA and IgE Fab molecular models with the IgG₃ Fab crystal structure. The three structures are superposed on their light chains (A), on their heavy chains (C). Light chain framework

(light green), heavy chain framework (dark green), CDRs (colored). The overall RMS deviations of framework residues are shown as RMS matrices (light chain- panel A and heavy chain- panel C), the overall RMS deviations of CDR residues are shown as RMS matrices (light chain- panel B and heavy chain- panel D). The RMS deviations of individual residues are plotted, panel A: light chain framework residues, panel B: light chain CDRs, panel C: heavy chain framework residues, panel D: heavy chain CDRs. The model on panel A covers both panel A and panel B results; the model on panel C covers both panel C and D results.

TABLE I. Exponential fit for IgA (A) and Sigmoidal fit for IgE Catalysis (B). Temperature for all experiments was 37°C.

(A) $I = I_0 * \exp(\text{Rate} * x)$ for decreasing, $I = I_0 * (1.0 - \exp(\text{Rate} * x))$ for increasing intensities.

(B) $I = (I_{\max} - I_{\min}) / (1.0 + \exp((t - t_{1/2})/\text{Width})) + I_{\min}$.

Table I. Cleavage Rate Data for IgE and IgA¹							
A	Peak	Rate (min ⁻¹)		RMSE	R ²		
IgA	M10	-6.267		0.501	0.986		
	L11	-5.548		0.615	0.966		
	M10'	-2.162		0.297	0.975		
	M10'''	-9.033		0.544	0.976		
B	Peak	I _{max}	I _{min}	Width(min)	T _{1/2}	RMSE	R ²
IgE	M10	44.29	12.58	16.39	57.64	0.62	0.989
	L11	45.08	13.83	14.95	64.64	0.61	0.986
	M10'	18.41	7.06	16.33	45.07	0.37	0.977
	M10'''	35.00	4.53	26.49	43.43	0.54	0.986

¹ Exponential fit for IgA (A) and Sigmoidal fit for IgE Catalysis (B). Temperature for all experiments was 37°C.

(A) $I = I_0 * \exp(\text{Rate} * x)$ for decreasing, $I = I_0 * (1.0 - \exp(\text{Rate} * x))$ for increasing intensities.

(B) $I = (I_{\max} - I_{\min}) / (1.0 + \exp((t - t_{1/2})/\text{Width})) + I_{\min}$.

Figure 1.

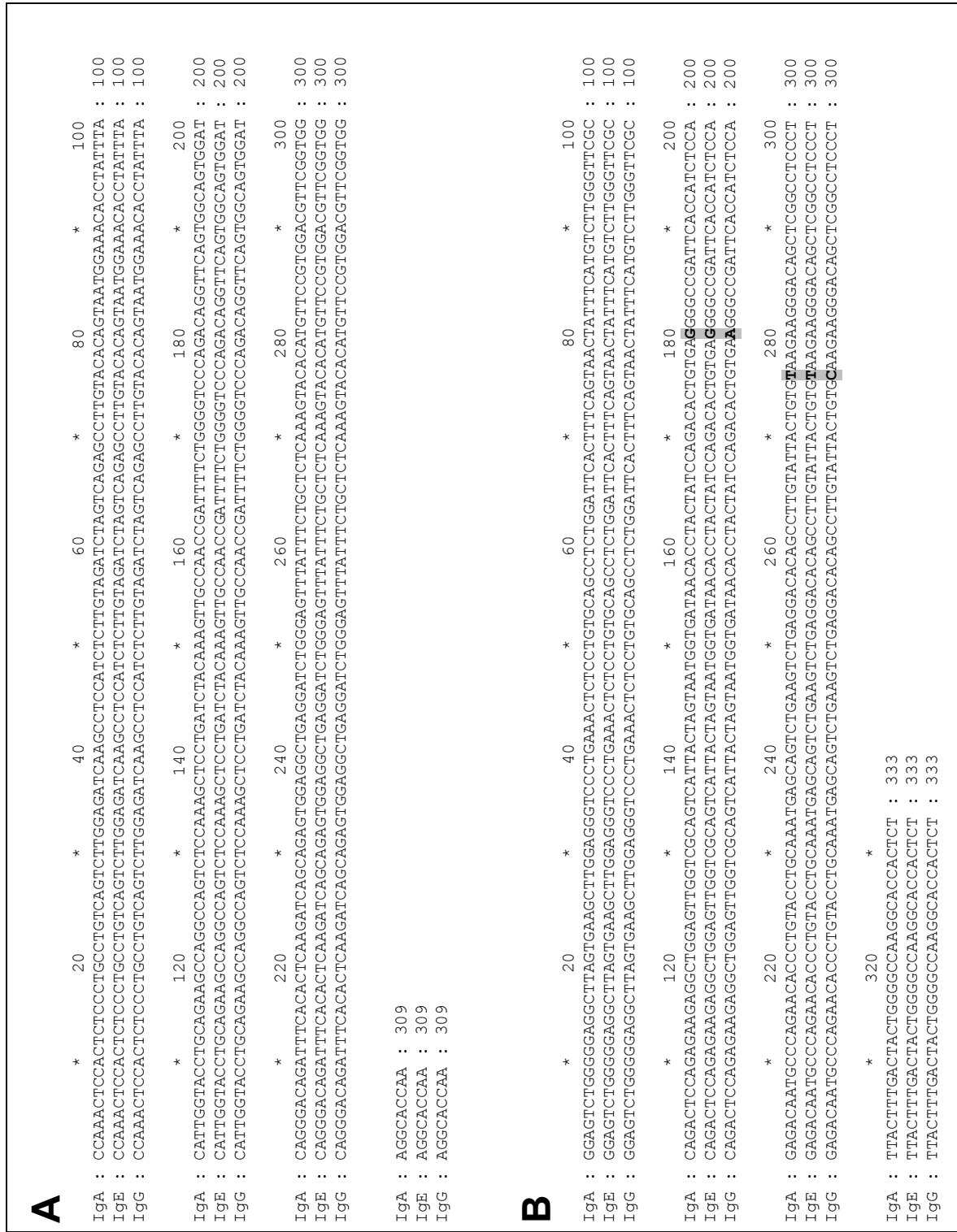


Figure 2.

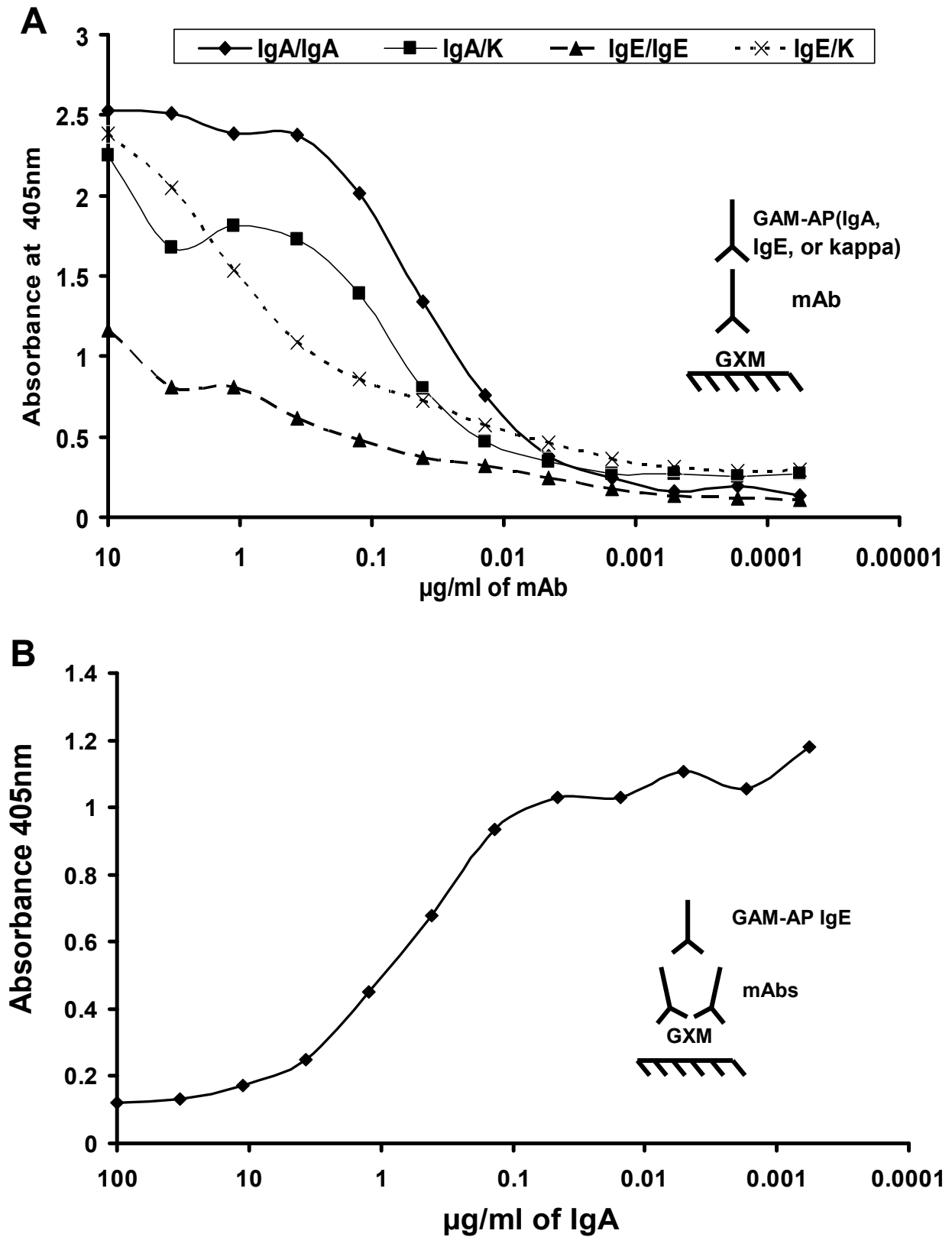


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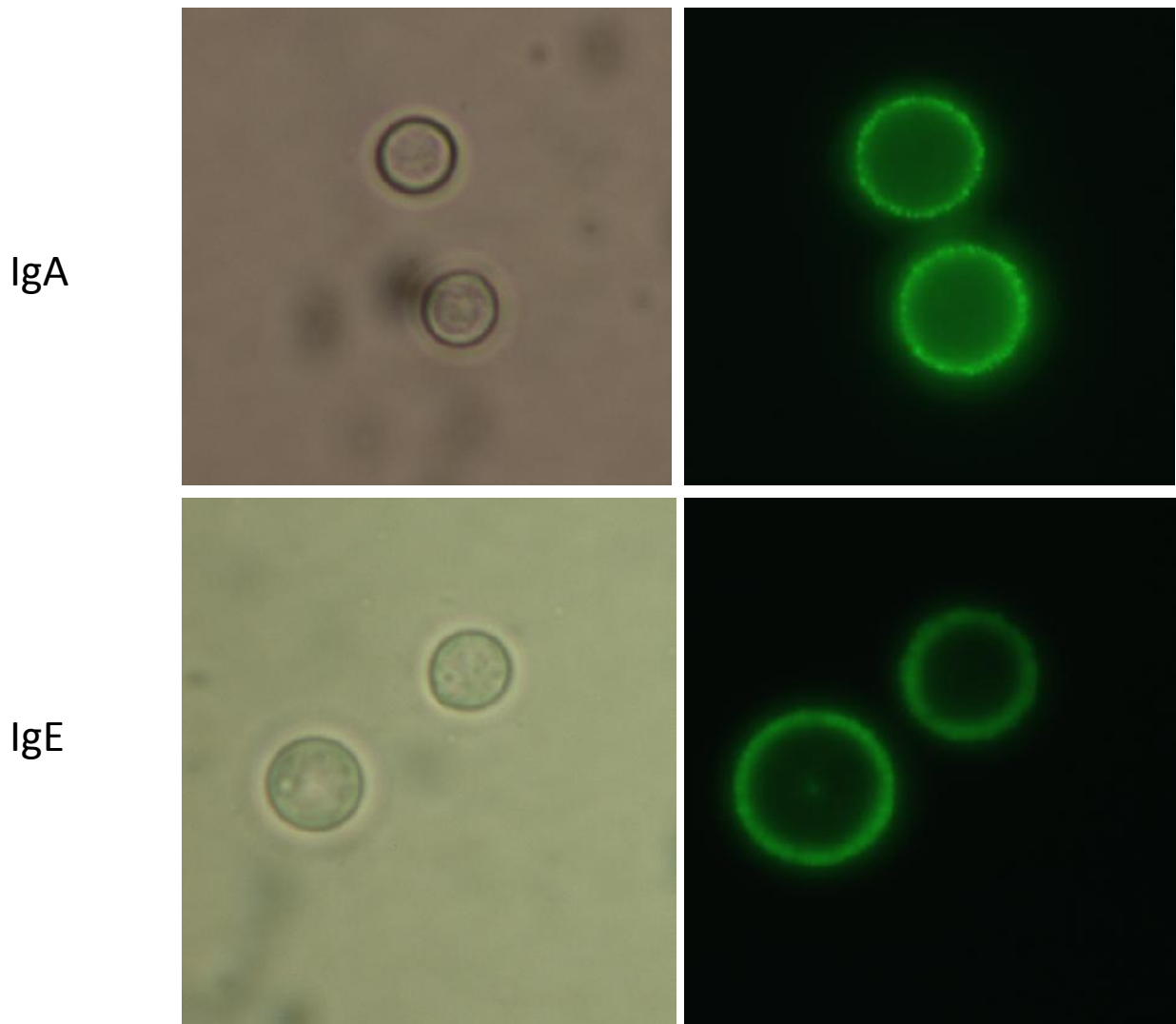
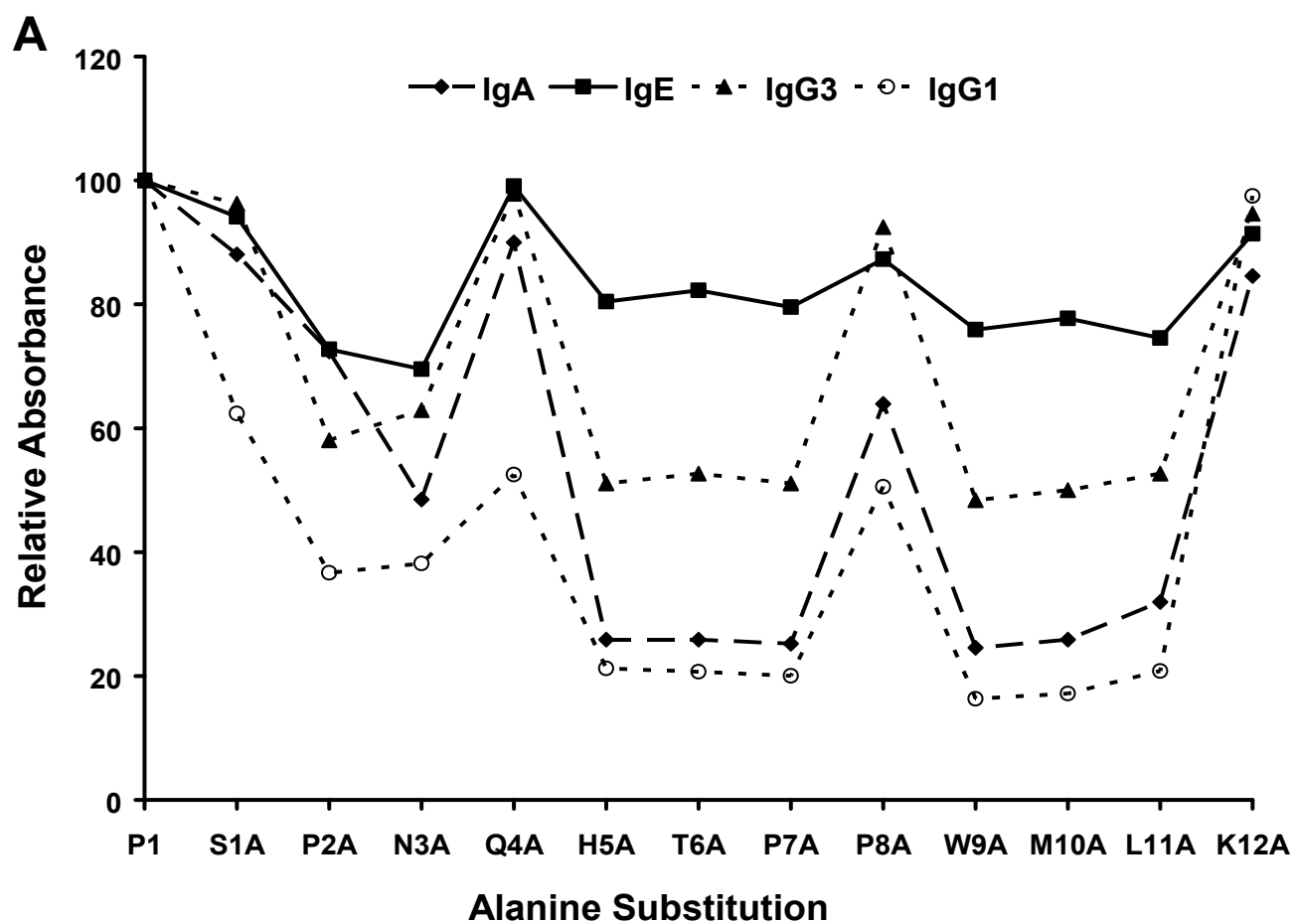


Figure 4.



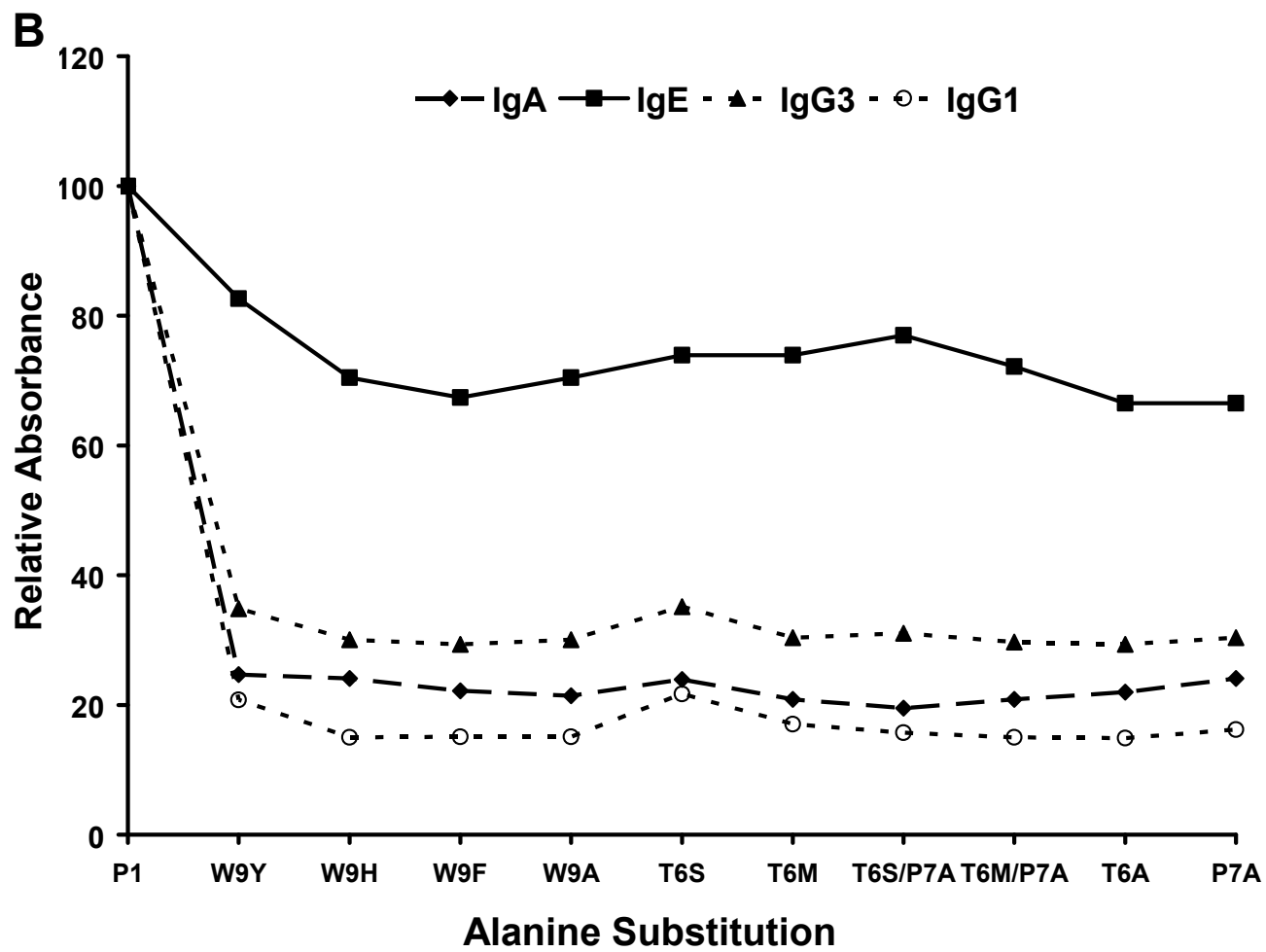


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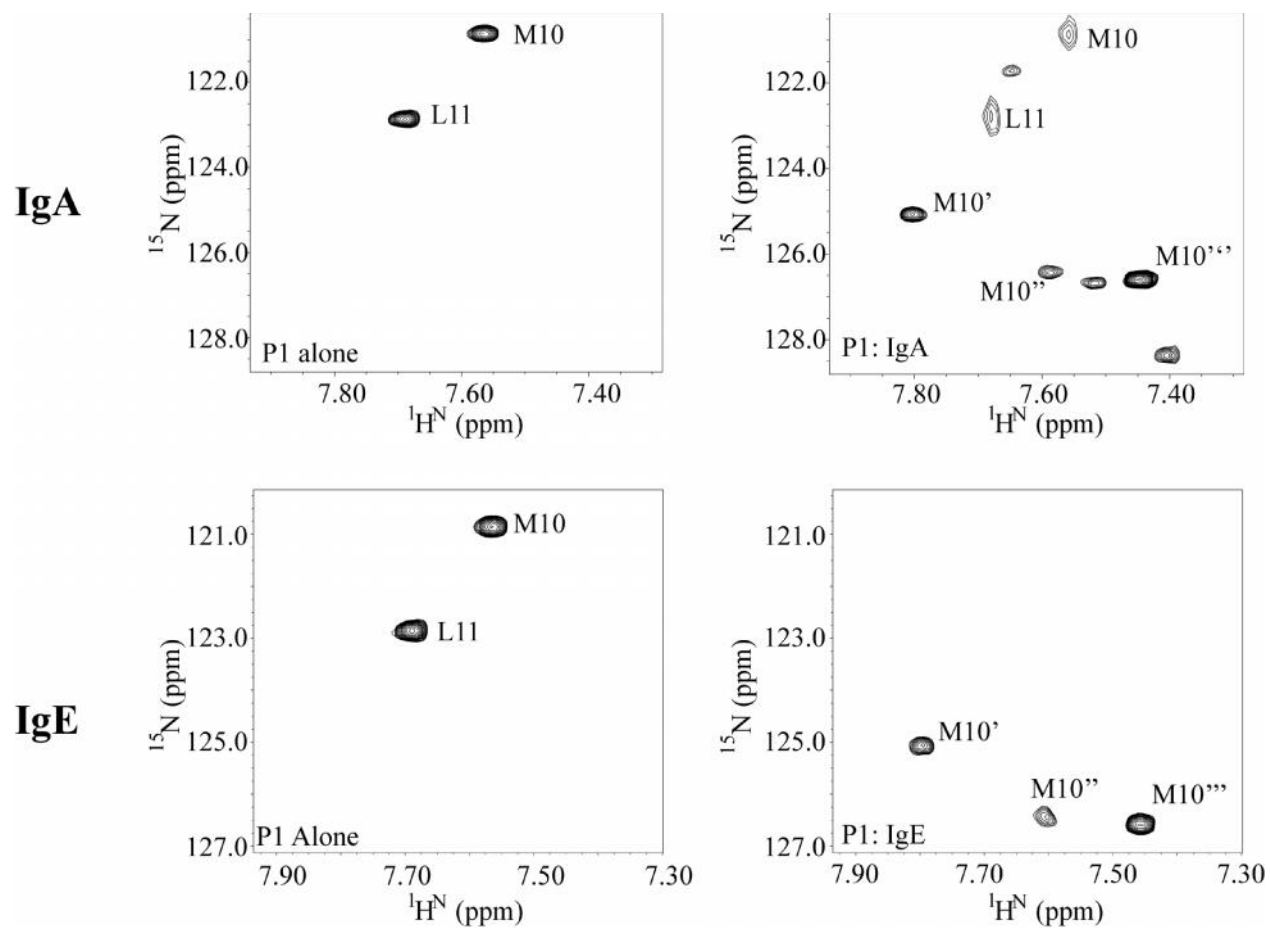


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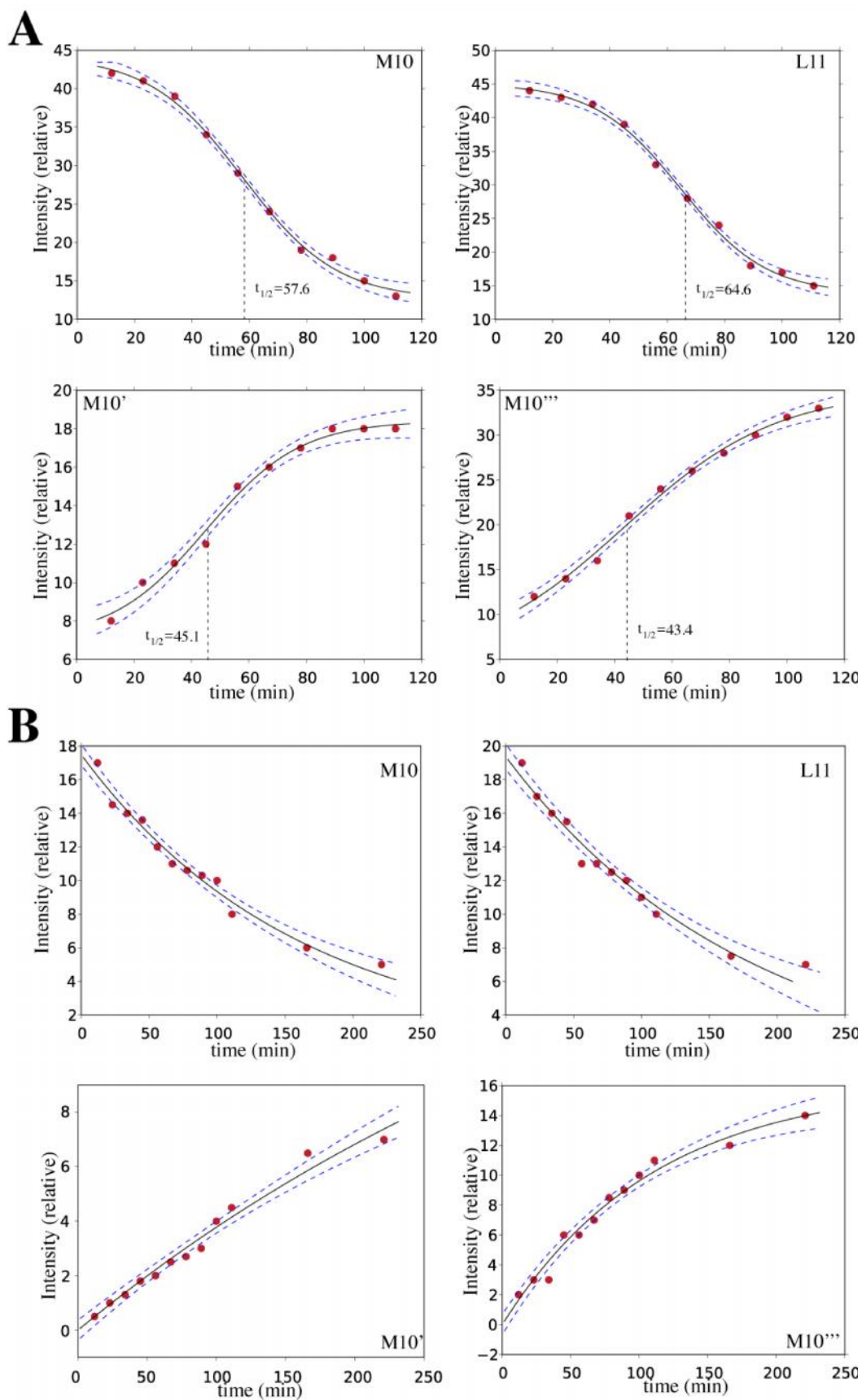
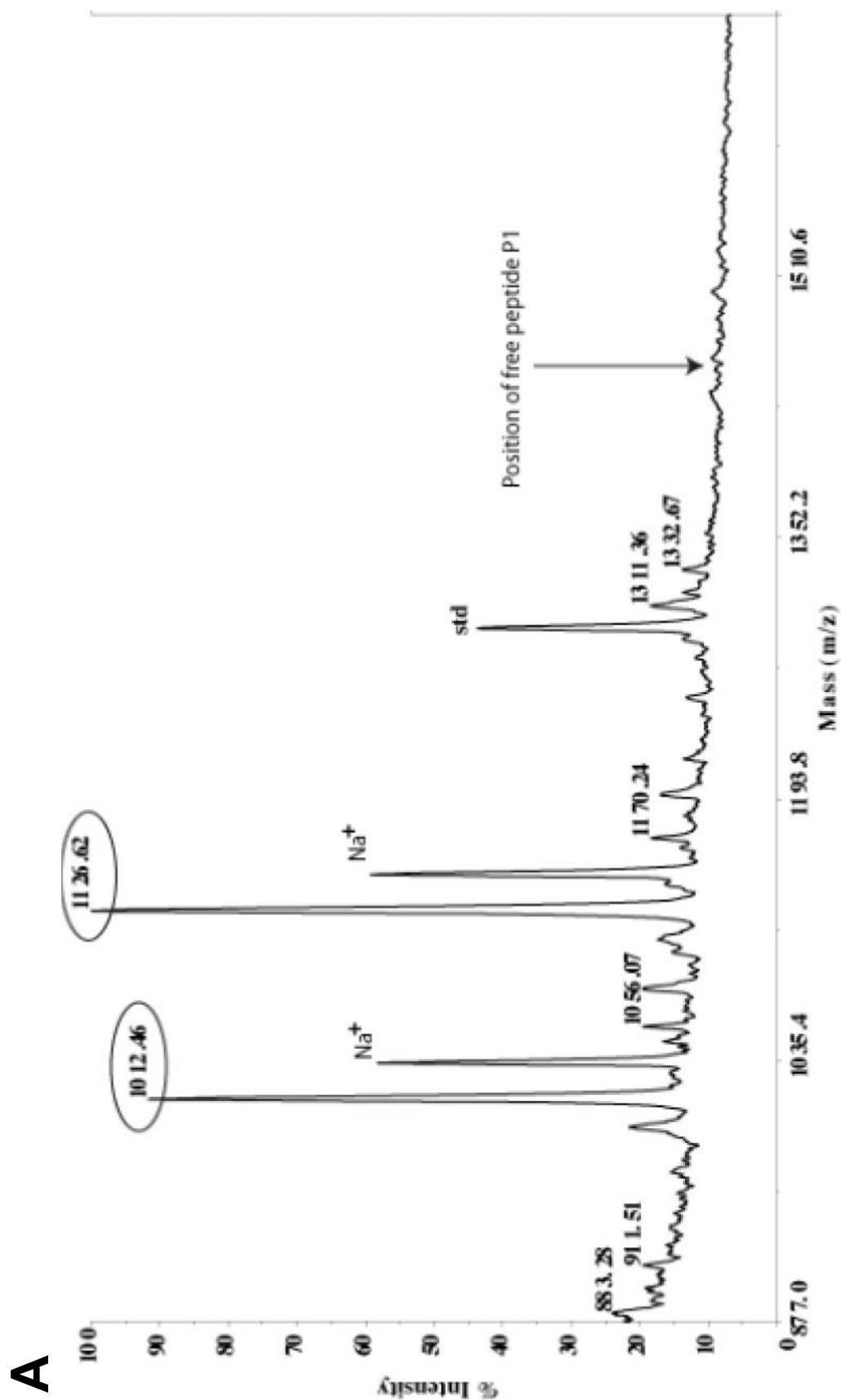


Figure 7.



B

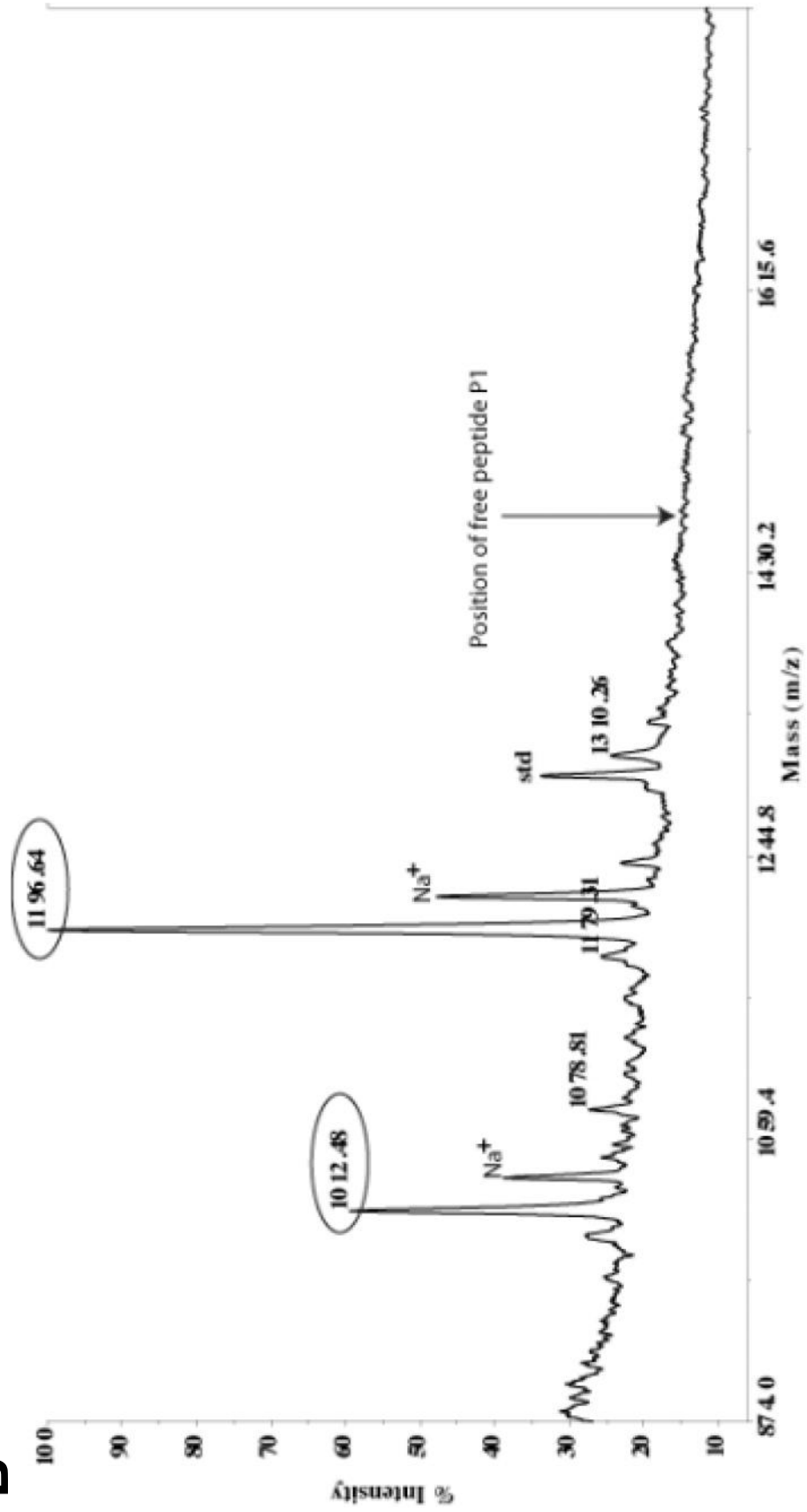
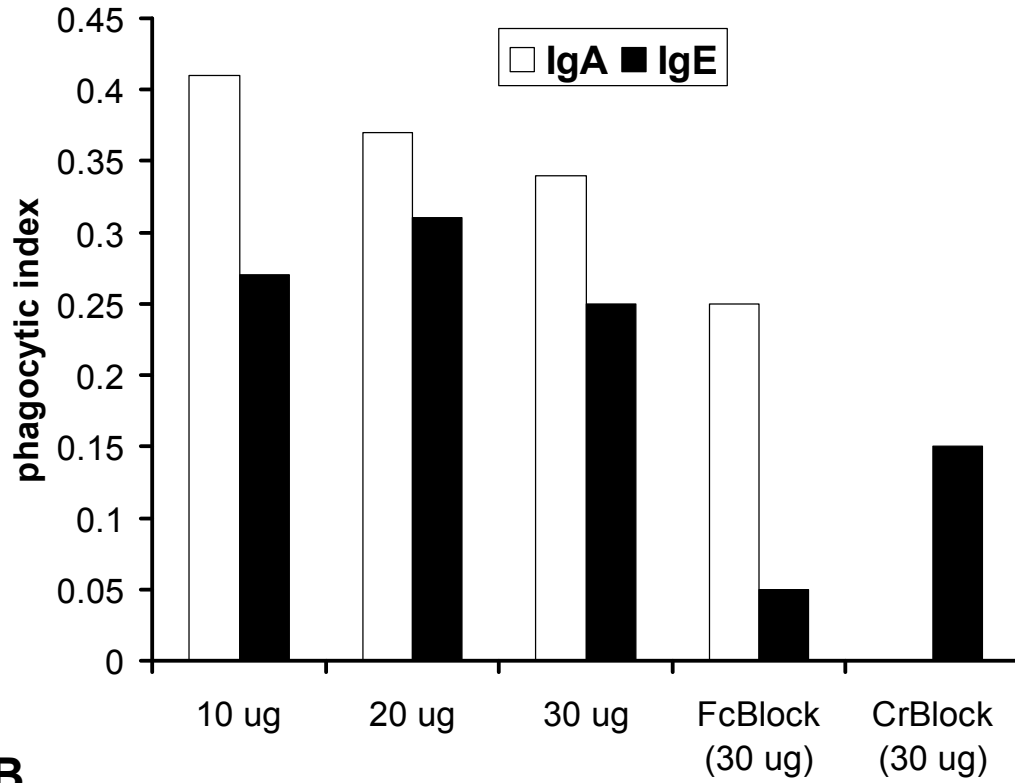


Figure 8.

A



B

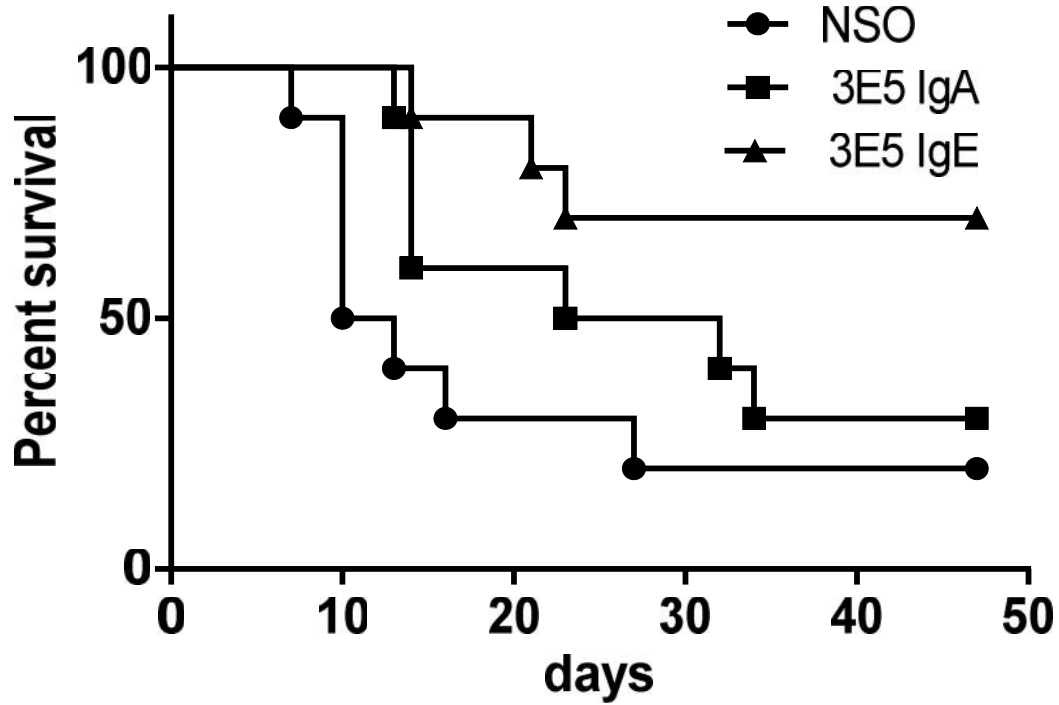


Figure 9.

