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

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Received for publication, October 19, 2014, and in revised form, February 17, 2015. Published, JBC Papers in Press, March 16, 2015, DOI 10.1074/jbc.M114.618975

Background: Ig µ, γ, and α constant regions can affect Ag specificity, but this is unknown for IgE.

Results: IgE and IgA variable regions differ in specificity and cleavage rates compared with each other and IgG subclasses.

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

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

In recent years several groups have shown that isotype switching from IgM to IgG to IgA can affect the affinity and specificity of antibodies 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 with 15N-labeled peptide mimetics of cryptococcal polysaccharide and found that these differed in specificity from each other. The IgE and IgA paratopes were probed by nuclear magnetic resonance spectroscopy with 15N-labeled peptide mimetics of cryptococcal polysaccharide antigen (Ag). IgE was found to cleave the peptide at a much faster rate than V 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 C 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 α and C regions affects their function and contributes to the special properties of those isotypes.

Since Porter’s classic studies in the 1950s (1), immunoglobulins (Ig) 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 antigen (Ag) specificity (for review, see Refs. 3 and 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 antibodies (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 idiotypic 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 Ag binding and 2) subclass-mediated differences in functional affinity leading to the recognition of new epitopes (6). The latter is 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 (10). Earlier studies show that creating a chimera from a human IgA C region and mouse V region alters Ag specificity (8). A different study comparing a human monomeric IgA1 and its IgG1 isotype revealed that although they bind the same epitope, these mAbs have significantly different binding affinities to the same Ag (9). Thus, like IgG isotypes, there is evidence that the IgA C region affects its Ag specificity.
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 (for review, see Ref. 18). In general, the IgM and IgG subclasses except for murine IgG2, 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, although 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 (FceR), 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 on 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 (C112, C113) (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 IgG2 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 Ag 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 IgG 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 Healthcare). The mAbs were then concentrated, and mAb concentration was determined by A280 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 (SPNQHT-PPW[15N]M[15N]LK).

Binding and Competition ELISAs—Binding experiments to GXM were tested by ELISA using methods previously described (24, 25). Briefly, 1 μg/ml 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-labeled goat anti-mouse antibody reagents (Southern Biotechnology, Birmingham, AL). Plates were developed with p-nitrophenol phosphate substrate (Sigma). All incubations were carried at 37 °C for 1 h, and 3 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 GXM and blocked, and both mAbs were added, keeping one Ab (10 μg/ml) at a constant concentration while changing the concentration of the second starting at 100 μg/ml diluted serially by three. The binding of the Ab kept constant was detected by adding alkaline phosphatase-labeled goat anti-mouse isotype IgE (10 μg/ml) secondary Ab and developed with p-nitrophenol phosphate substrate (1 mg/ml). All incubations, washes, and absorbance measurements were performed as mentioned above.

VH and VL Sequence Analysis—The VH and VL sequences of the 3E5 IgG subtypes were determined previously (26) and are available in GenBank™ (accession numbers AY674854.1, AY674857.5, 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, GATGTTGTGTAGTGCAC-CCAAACTCCACT; 3E5VL-R, GATTTCCAGCTTGGTGAGTGGTC; 3E5VH-F, AGCTGAAGCTGTGGAGTCTGG; 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 isotype-specific Ab (10 μg/ml). After this incubation, cells were washed and resuspended in 0.1M 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 3 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 units/ml IFN-γ and 1 μg/ml 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 3 times in triplicate, 100 macrophages with ingested or non-ingested crypto were counted per well, and the results were reported as percent phagocytosis.

Survival studies—6–8-Week old BALB/c mice were used in survival experiments. 100 μg of either 3E5 IgA or IgE mAb were injected intraperitoneally 2 h before infecting with 1 × 10^6 C. neoformans cells intravenously via tail vein. Survival was monitored daily.

NMR Spectroscopy—IgE was concentrated to 32 μM in 0.1M Bis-Tris and 0.15M NaCl, pH 6.5, buffer. 100 μM P1 (Chem Pep) was added just before NMR analysis for the 37 °C experiments. 1H, 15N 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.

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.
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 the 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 Generalized Born/Volume Integral 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, whereas 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 VH and VL of the 3E5 IgE and IgA were sequenced. The VL sequences of the IgA and IgE variants were found to be identical to all of the IgG subtypes. However, for the VH sequences we found two polymorphisms distinguishing the IgA and IgE variants from the IgG variants (Fig. 1). These polymorphisms presumably arose during the isotype switching process and result in two amino acid differences in the VH; position 65 Lys → Arg, located in a buried region between complimentarity-determining region (CDR) 2 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 (Fig. 2A) and competed with one another (Fig. 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, whereas IgA had a more clustering or punctuate binding pattern (Fig. 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 (Fig. 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 with the other isotypes (Fig. 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]Met-10-[15N]Leu-11-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 HSQC and mapped chemical shift
FIGURE 6. Proteolysis of peptide P1 by IgE and IgA at 37 °C. Proteolytic activity of IgE (A) and IgA (B) was analyzed by fitting the observed NMR intensities of $[^{15}\text{N}]$Met-10 and $[^{15}\text{N}]$Leu-11. IgE and IgA show different catalytic mechanisms evidenced by different intensity fits. Intensities arising from IgE catalysis fit well into a Boltzmann Sigmoid, suggesting an allosteric mechanism regulating P1 proteolysis, whereas intensities from IgA catalysis fit to an exponential function indicating a more simple proteolytic mechanism. Met-10 and Leu-11 indicate Met-10 and Leu-11 of the free peptide, whereas Met-10$''$ and Met-10$'''$ indicate Met-10 from cleaved P1 fragments.
IgE and IgA Isotypes Have Differences in Specificity

TABLE 1
Exponential fit for IgA
The temperature for all experiments was 37 °C. $I = I_o \times \exp(\text{rate} \times x)$ for decreasing intensities, $I = I_o \times (1.0 - \exp(\text{rate} \times x))$ for increasing intensities. RMSE, root-mean-square error.

| Peak  | Rate $min^{-1}$ | RMSE | $R^2$ |
|-------|-----------------|------|--------|
| Met-10       | −6.267          | 0.501 | 0.986  |
| Leu-11       | −5.548          | 0.615 | 0.966  |
| Met-10'      | −2.162          | 0.297 | 0.975  |
| Met-10''     | −9.033          | 0.544 | 0.976  |

TABLE 2
Sigmoidal fit for IgE catalysis
The temperature for all experiments was 37 °C. $I = (I_{max} - I_{min})/(1.0 + \exp((t - t_{1/2})/width)) + I_{min}$. RMSE, root-mean-square error.

| Peak  | $I_{max}$ | $I_{min}$ | Width | $t_{1/2}$ | RMSE | $R^2$ |
|-------|-----------|-----------|-------|-----------|------|-------|
| Met-10       | 44.29     | 12.58     | 16.39 | 57.64     | 0.62 | 0.989 |
| Leu-11       | 45.08     | 13.83     | 14.95 | 65.44     | 0.61 | 0.986 |
| Met-10'      | 18.41     | 7.06      | 16.33 | 45.07     | 0.37 | 0.977 |
| Met-10''     | 35.00     | 4.53      | 26.49 | 43.43     | 0.54 | 0.986 |

The proteolytic activity of both IgE and IgA was investigated by analyzing NMR peak intensities of labeled amino acid Met-10 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 (Fig. 5). The disappearance of bound P1 resonances and appearance of new resonance peaks were time-dependent, suggesting that the peptide was being modified and perturbations. We measured the $^{15}$N and $^1$H$^{15}$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 IgG2b), 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 $^{15}$N-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 $^{15}$N-labeled amide bond on either Met-10 or Leu-11.

FIGURE 7. IgA and IgE 25 °C mass spectrometry results. A, IgA + P1 fragmentation, the appearance of two new peaks at 1012 and 1126 m/z is seen after 3 h. B, IgE + P1 fragmentation, two new peaks at 1012 and 1196 m/z are seen after 3 h.
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 (Fig. 6). The cleaved Leu-11 peak is not visible in IgE complex, suggesting the Met-10–Leu-11 peptide bond is targeted by this mAb. In the peptide-IgA complex, extra peaks were visible, and at least one fragment still possessed the \[^{15}N\]Leu-11 residue, indicating the Met-10–Leu-11 peptide bond was still intact (Fig. 5). The fits and rates are summarized in Tables 1 and 2. The HSQC spectra of P1 alone were also collected at 37 °C, and 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 masses (m/z) of ~1196 (SPNQHTPPWM/LK) and ~1012 (SP/NQHTPPWM/LK), confirming proteolysis of the peptide (Fig. 7A). Mass spectrometric analysis of the IgA+P1 solution after NMR analysis at 37 °C revealed the appearance of two fragments with the masses (m/z) of ~1126 (SP/NQHTPPWML/K) and ~1012 (SP/NQHTPPWM/LK) (Fig. 7B). As a positive control, we have seen the same phenomenon with similar if not identical P1. The HSQC spectra of P1 alone were also collected at 37 °C, and resonance peaks did not change, and there were no visible new peaks, suggesting that P1 is stable in the buffer used (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, whereas blocking CR completely abrogated the opsonic capacity of IgA (Fig. 8A). Administration of either 3E5 IgE or IgA to mice before infection with *C. neoformans* prolonged survival (Fig. 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 ID 4HDI) (15) on either their light chains (Fig. 9, A and B; RMSD of 0.873 Å) or their heavy chains (Fig. 9, C and D, RMSD of 0.678 Å). The constant regions influence the framework conformations and both the light chain and heavy chain CDRs (Fig. 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 after 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 α and ε 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 ε 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), which had already provided us with a set of V region IgG subclasses. However, the Ig gene sequences of the 3E5 IgA and IgE clones were found to have different amino acids in the VH at positions 65 and 97. Position 97 is in the CDR3 and, therefore, is 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 IgGs 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 (Fig. 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 because 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 ε C region. Consequently this Ab set provides us with the opportunity to explore whether the ε C region, like the γ and α C regions, can affect Ab specificity.

In early studies we noted that V-region identical Abs differing in the C region produced subtle differences in IF pattern when bound to the C. neoformans capsule (8, 40). In fact, it was differences in the IF patterns 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. Because 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 mimic for fine dissection of specificity.

**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) and on their heavy chains (C). Light chain framework (light green), heavy chain framework (dark green), and CDRs (colored) is shown. The overall RMS deviations of framework residues are shown as RMS matrices (panel A, light chain; panel C, heavy chain), the overall RMS deviations of CDR residues are shown as RMS matrices (panel B, light chain; panel D, heavy chain). The RMS deviations of individual residues are plotted. Panel A, light chain framework residues. **Panel B**, heavy chain framework residues. **Panel C**, Light 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.
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 C 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]Met-10–[15N]Leu-11-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: Leu-11–Lys-12, 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. Because 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 promotes 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 FcγR, there is evidence that it can also interact with the FcγR (43). Furthermore, IgE and IgG immune complexes have been shown to trigger a similar reaction from macrophages (44). Passive administration of 3E5 IgA and IgE to mice before 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 C 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 classic 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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