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DETERMINATION OF A LINEAR B-CELL EPITOPE IN EQUINE IGG3 ANTIBODIES FOR IMPROVED DETECTION IN THERAPEUTIC PREPARATIONS

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Abstract- Equine immunoglobulins (eqIg) and its fragments, $F(ab')_2$ and F(ab'), are normally employed as immunopharmaceuticals for several diseases, like treatment of snake bits and neutralizing bacterial toxins. However, it can induce serious side effects in a subset of patients, specifically hypersensitivity from their immune response against whole eqIg contaminants. Therefore, the important antigenic determinants in equine heavy chain IgG3 (eqIgG3) were defined by a library of overlapping 15-mer peptides that covered the entire amino acid sequence (354 residues) and were immobilized onto a membrane. Screening with rabbit sera identified eleven distinct epitopes in eqIgG3 that were recognized by rabbit antibodies. The performance of an antisera against the most prominent epitope was analyzed by ELISA and a competitive assay. The results suggest an effective antisera to detect whole, undigested eqIg within equine sera preparations for minimizing adverse side effects and support this methodology for producing targeted antibodies.
Keywords- Equine immunoglobulin, Peptide array, SPOT-synthesis, B-epitopes, Synthetic peptides

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Introduction

Antibody preparations from hyperimmune equine serum are currently used as prophylactics in a variety of medical emergencies [1]. Uses of purified therapeutic serum include treatments for diphtheria and tetanus [2]; botulism and rabies [3]; snake venom [4]; as well as bites from spiders, scorpions and others [5,6]. While extremely effective, equine immunoglobulin (eqIg) preparations can induce anaphylactic reactions in patients. The risk varies greatly in preparations between producers from differences in purity and antibody aggregation [2,7-9]. Anaphylaxis can result from the binding of the Fc domain in eqIg to receptors on human cells [10]. The use of the F(ab)´or $F(ab)'_2$ fragments minimizes risk, but the presence of intact eqIg often persists and their absolute quantities need to be determined [11,12]. The method of immunochromatography using Fc specific antibodies is a quickly and easy method, but is dependent on the quality of the anti-Fc antibodies employed [13]. Currently, specific monoclonal antibodies (mAbs) are available, but their antigenic determinants are not known [14,15].

The major antibody class in horses is IgG, which have seven constant region genes, the greatest number among all mammals [16]. Here, we have targeted eqIgG3, the second most common antibody in the sera [17] of horses and the major isotype involved in immunorecognitions of snake venom toxins [18], for determining the epitopes recognized by rabbit antibodies with the goal of identifying the most prominent antigens for generating targeted anti-eqIgG3 antibodies. The recognition of specific regions in a protein is often found advantageous for diagnostic purposes and the selection of the correct peptide sequence for antibody production is essential. Using the parallel synthesis of peptides on cellulose membrane (SPOT-synthesis), we report the major equine IgG3 epitope sequences. Furthermore, an antiserum was produced against the most prominent and its prospects for use in ELISA-based quantitative studies are presented.

Materials and Methods

Reagents and Antibodies

The amino acids for peptide synthesis were from Calbiochem-Novabiochem Corp. (Germany) and the CLC-ODS HPLC column from Shimadzu (Kyoto, Japan). Super SignalR West Pico chemiluminenscent substrate was from Pierce Biotechnology (Rockford, IL, USA). Amino-PEG_{500}-UC540 cellulose membranes were obtained from Intavis AG Bioanalytical Instruments (Germany). Pyperidine, acetonitrile and trifluoracetic acid were from Fluke. A rabbit anti-equine immunoglobulin serum, peroxidase-labelled sheep anti-rabbit polyclonal serum, bovine serum albumin, purified equine immunoglobulin (hIg), 3,3,5,5`tetramethylbenzidine (TMB) and

Tween-20 were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Centricon 10 filters from Amicon (CA, U.S.A.). CDP-Star® Substrate was from Applied Biosystems (USA). Sequence reagents and all other reagents and chemicals were from Merck (Darmstadt, Germany). Tetanus toxoid (TT) was a kind gift from Butantan Institute (São Paulo, Brazil).

Synthesis of the Cellulose-membrane-bound Peptide Array

Sixty-nine consecutive overlapping peptides [Fig-1C] were designed to represent the entire 354 amino acids sequence of the equine IgG3 protein. These peptides were 15 amino acids in length with an offset of five amino acids from the previous peptide and automatically prepared on cellulose membranes according to standard SPOT synthesis protocols [19] using an Auto-Spot Robot ASP-222 (Intavis Bioanalytical Instruments, Koeln, Germany). Coupling reactions were followed by acetylation with acetic anhydride (4%, v/v) in *N*-*N*-dimethyformamide to render peptides unreactive during the subsequent steps. Following acetylation, the Fmoc protective groups were removed by the addition of piperidine to render nascent peptides reactive. The additional amino acids were added sequentially by repeating the process of coupling, blocking and deprotection. The last step of synthesis was the deprotection of the amino acid side chains using a solution of dichloromethane-trifluoracetic acid-triisobutylsilane (1:1:0.05, v/v/v) and washed with methanol. The resulting membranes supporting the array of synthetic peptides were either probed immediately or stored at -20°C until needed. Negative controls consisted of spots without peptide and the peptide IHLVNNESSEVIVHK (*Clostridium tetani* precursor) peptide. Positive controls were also included in each assay.

Screening of SPOT Membranes

SPOT membranes were washed with TBS (50 mM Tris-buffer saline, pH 7.0) and then blocked with TBS-CT (Tris-buffer saline, 3% casein, 0.1% Tween-20, pH 7.0) at room temperature under agitation or overnight at 4°C. After extensive washing with TBS-T (Tris-buffer saline, 0.1% Tween-20, pH 7.0), membranes were incubated for 2h with rabbit sera anti-equine IgG (1:250) in TBS-CT and washed again with TBS-T. After that, membranes were incubated with sheep alkaline phosphatase labeled anti-rabbit IgG (1:5000 in TBS-CT) for 1h, then washed with TBS-T and followed with an incubation in CBS (50 mM citrate-buffer saline, pH 7.0). Chemiluminenscent CDP-Star® Substrate (0.25 mM) with Nitro-Block-II™ Enhancer (Applied Biosystems, USA) was added to complete the reaction.

Scanning and Measurement of Spot Signal Intensities

Chemiluminescent signals were detected on MF-ChemiBis 3.2 (DNR Bio-Imaging Systems, Israel). A digital image file was generated at a resolution of 5 MP, which was used in association with TotalLab Software (Nonlinear Dynamics, USA). Signal intensities were quantified with TotalLab Software using algorithms that compared the intensity between background, spot area, negative control to define the empirical probability that signal at a spot was distinct from the background signal.

Standard Solid-phase Peptide Synthesis

Soluble peptides were synthesized at a 50 nmol scale on a multiple synthesizer PSS-8 (Shimadzu, Kyoto, Japan) according to the standard Fmoc machine protocol using Merrifield resin and PyBOP activation. All peptides were analyzed by reverse phase HPLC (CLC-6B, Shimadzu) on a Vydac C18 column using a linear gradient of 5-60% acetonitrile/water (0.05% trifluoracetic acid) generated over 20 min with a flow rate of 1.2 ml/min and detection of peptides at 214 nm and by MALDI-MS (Bruker Daltonics, New Jersey) using a-cyano-4-hydroxy-cinnamic acid as the matrix. Before use, peptides were purified to >95% purity by preparative HPLC on a Vydac C18 column.

Peptide-tetanus Toxoid (TT) Conjugation

The synthetic (CGGTWYVDGTEVKTAKTMGG) peptide was coupled to TT using 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) [20]. TT (25 mg/ml) was first linked to the peptides (5 mg/ml) and afterwards to the carrier (10 mg/ml) in order to favor linkage by the amino-terminal group of the peptide. Coupling efficiency was tested by filtrating the reaction mix through Millipore Centricon® Centrifugal Filter Units (cut-off 10 kDa). Peptide conjugate concentration was determined using a QuBit devise (GE Healthware). The efficiency was calculated as the quantity of peptide placed into the reaction minus the quantity of peptide after filtration divided by quantity of starting peptide. In each case, efficiency was between 80 and 85%.

Preparation of Anti-peptide Antibodies

Polyclonal antibodies were raised in rabbits by subcutaneously injecting the conjugate (500 µg) emulsified with complete Freund' adjuvant for the 1st injection followed by emulsification with incomplete Freund's adjuvant for the 2nd and 3rd injections) Freund's adjuvant. Blood was collected from the animals at regular intervals and five days after the last booster injection.

CELLU SPOT Assay

The presence of peptide specific antibodies was analyzed by dot blot through immobilization of the peptide-TT (1 µg) and TT alone (1 µg) using a robotic Auto-Spot SL (Intavis Bioanalytical Instruments, Koeln, Germany) onto cellulose membranes. After drying, the membrane was blocked overnight with 1% bovine albumin in TTBS buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% v/v Tween-20) followed by two 5 min washes with TTBS. After incubation with the indicated dilution of anti-peptide antibody for 1 h, membranes were washed twice with TTBS for 10 min and treated with 1:8000 dilutions in TTBS of 1 mg/ml of goat anti-rabbit Ig-peroxidase conjugated for 1 h at room temperature. After three washes with TTBS and one with TBS, peroxidase activity was revealed using enhanced chemiluminescence with SuperSignalR WestPico Chemiluminescent Substrate according to manufacturer's instructions. Signals were detected on a MF-ChemiBis 3.2. Controls consisted of probing without anti-peptide antibodies and using pre-immunization sera, which showed no signals (data not shown).

Direct and Indirect ELISA Assay

Aliquots (50 µl) of chromatographically purified eqIg (3 µg/ml in PBS) prepared in fresh 0.1 M sodium carbonate buffer (pH 9.6) were added to each well of an ELISA plate (MaxiSorp™, Nunc, Roskilde, Denamark) and incubated at 4°C overnight. Next, plates were washed with PBS 3 times followed by blocking with 1% BSA in PBS at 37°C for 2h. The plates were washed more three times with PBS and used directly to detect the presence of antibodies in the serum of SP-immunized rabbits (direct ELISA) or incubates with a mixture of SP-serum of immunized rabbits (indirect ELISA). Peptides of the indicated concentrations (1-70 ng/ml in PBS) were incubated 1h at 37°C with samples of rabbit anti-SP serum (50 µl, diluted 1:100 in PBS) in eppendorf tubes before adding to the hIg-

coated wells. After 2h at room temperature bound rabbit anti-SP antibodies were detected with a goat anti-rabbit Ig conjugated with HRP (Sigma). Competition (percent inhibition of binding) was calculated considering 100% as the OD_{405} of rabbit anti-whole eqIg.

Epitope Prediction, Bioinformatics Analysis and IgG3 Heavy Chain Model

Predictions of epitopes in eqIgG3 were performed with Kolaskar Tongaonkar and Bepipred antigenic scales (http://tools.immuneepitope.org/tools/bcell/iedb_input). Predicted secondary structures for eqIgG3 were from the PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred). An analysis of potential three-dimensional structures for domains in eqIg were executed through the Blast-P program [21] against structures deposited in the database of protein-PDB (http://www.pdb.org/pdb/ home/home.do). An eqIgG3 heavy chain model was generated by homology using human IgG as a template (PDB H1Z). Sequences were manually aliened and the quality of the model was evaluated using PROCHEK [22] and VERIFY 3D [23] online (http://nihserver.mbi.ucla.edu/SAVES/).

DNA sequences representing the different genetic variants of constant regions in equine (AJ302055; AJ302056; AJ302058; AJ312379; AJ312380; AJ312381) and rabbit (K00752) immunoglobulin heavy chains were retrieved from the National Center for Biotechnology Information database.

Results

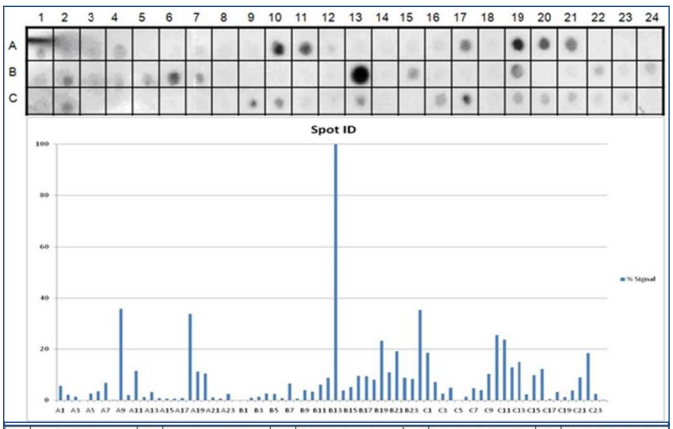
Identification and Mapping using Synthetic Peptides of Linear Epitopes of the Heavy Chain Equine Immunoglobulin

The epitopes present in the CH1, CH2 and CH3 domains of the equine immunoglobulin heavy chain were mapped using a parallel Spot-synthesis strategy to identify candidate epitopes for generating targeted antibodies. To define these epitopes precisely, we designed and synthesized 69 overlapping, 15-mer peptides that spanned the entire amino acid sequence of eqIgG3 with an offset of 5 amino acid between peptides. The library of peptides were used to evaluate the binding activity of a commercially available rabbit anti-eqIg. An image of the pattern of recognition from a representative experiment is shown in the top panel of [Fig-1], which displays results identical to those of three independent assays**.** By plotting the measured intensities, approximately 45 positive spots were identified [Fig-1, middle panel]. By comparing the amino acid composition of the positive peptides located at each position [Fig-1, lower panel], eleven candidate immunodominant antigenic determinants were defined.

Table 1- Epitopes in eqIgG3 identified by rabbit IgG with their amino acid sequence and domain position

Epitope	IgG-Sequence	Ig Region
eqIg-1	^{6}PKVFPLAPSC^{14}	F(ab)'2
eqIg-2	^{46}LTSGV^{50}	F(ab)'2
eqIg-3	^{67}SMVTVAPAS^{75}	F(ab)'2
eqIg-4	^{81}YICNVAHPASS^{91}	F(ab)'2
eqIg-5	^{126}PCPCECPKCPA^{136}	Fc(hinge)
eqIg-6	^{151}KPKDV^{155}	Fc(C_{H2})
eqIg-7	^{183}YVDGTEVKTAKTM^{195}	Fc(C_{H2})
eqIg-8	^{208}SVLRIQH^{214}	Fc(C_{H3})
eqIg-9	^{291}HPEPEGKYRT^{300}	Fc(C_{H3})
eqIg-10	^{321}TDRWQQGTTFTCV^{333}	Fc(C_{H3})
eqIg-11	^{340}HNHVMQ^{345}	Fc(C_{H3})

Their core amino acids, which consisted of 6-15 amino acids, are listed in [Table-1]. The antigens were named eqIg1-7 sequentially from the amino terminus to the carboxyl terminus. Based on their positions in the heavy chain sequence, four (hIg1-eqIg4) of the candidate antigenic determinants were located in the F(ab`2) region, one in the hinge (eqIg5), two (eqIg6-7) in the CH2 domain and four (eqIg8-eqIg11) in the CH3 domain.



A1	ASTTAPKVFPLAPSC	A15	VPASSLESKTYICNV	B5	GGPSVFIFPPKPKDV	B19	LRIQHQDWLNGKKFK	C9	DITVEWQSNEHPEPE
A2	PKVFPLAPSCGTTSD	A16	LESKTYICNVAHPAS	B6	FIFPPKPKDVLMITR	B20	QDWLNGKKFKCKVNN	C10	WQSNEHPEPEGKYRT
A3	LAPSCGTTSDSTVAL	A17	YICNVAHPASSTKVD	B7	KPKDVLMITRTPEVT	B21	GKKFKCKVNNQALPA	C11	HPEPEGKYRTTEAQK
A4	GTTSDSTVALGCLVS	A18	AHPASSTKVDKRIEP	B8	LMITRTPEVTCLVVD	B22	CKVNNQALPAPVERT	C12	GKYRTTEAQKDSDGS
A5	STVALGCLVSSYFPE	A19	STKVDKRIEPVLPKP	B9	TPEVTCLVVDVSHDS	B23	QALPAPVERTISKAT	C13	TEAQKDSDGSYFLYS
A6	GCLVSSYFPEPVTVS	A20	KRIEPVLPKPTTPAP	B10	CLVVDVSHDSSDVLF	B24	PVERTISKATGQTRV	C14	DSDGSYFLYSKLTVE
A7	SYFPEPVTVSWNSGT	A21	VLPKPTTPAPTVPLT	B11	VSHDSSDVLFTWYVD	C1	ISKATGQTRVPQVYV	C15	YFLYSKLTVETDRWQ
A8	PVTVSWNSGTLTSGV	A22	TTPAPTVPLTTTVPV	B12	SDVLFTWYVDGTEVK	C2	GQTRVPQVYVLAPHP	C16	KLTVETDRWQQGTTF
A9	WNSGTLTSGVRTFPS	A23	TVPLTTTVPVETTTP	B13	TWYVDGTEVKTAKTM	C3	PQVYVLAPHPDELSK	C17	TDRWQQGTTFTCVVM
A10	LTSGVRTFPSVLQSS	A24	TTVPVETTTPPCPCE	B14	GTEVKTAKTMPNEEQ	C4	LAPHPDELSKNKVSV	C18	QGTTFTCVVMHEALH
A11	RTFPSVLQSSGLYSL	B1	ETTTPPCPCECPKCP	B15	TAKTMPNEEQNNSTY	C5	DELSKNKVSVTCLVK	C19	TCVVMHEALHNHVMQ
A12	VLQSSGLYSLSSMVT	B2	PCPCECPKCPAPELL	B16	PNEEQNNSTYRVVSV	C6	NKVSVTCLVKDFLPT	C20	HEALHNHVMQKNVSH
A13	GLYSLSSMVTVPASS	B3	CPKCPAPELLGGPSV	B17	NNSTYRVVSVLRIQH	C7	TCLVKDFLPTDITVE	C21	HNHVMQKNVSHSPGK
A14	SSMVTVPASSLESKT	B4	APELLGGPSVFIFPP	B18	RVVSVLRIQHQDWLN	C8	DFLPTDITVEWQSNE		

Fig. 1- Reactivity of the rabbit sera immunized against eqIgG to cellulose-bound peptides. The top panel shows the chemiluminescence image of a representative membrane spotted with the 15-mer peptide library representing eqIgG3 reacted against a commercial rabbit sera directed against eqIgG. The middle panel graphs the measured intensity from the chemiluminescence in the top panel. The lower panel lists the position and amino acid sequence of each peptide in the library. C22-C24 are negative controls.

Epitope Prediction

Epitope prediction is an important tool and is based on protein structure properties, like accessibility, flexibility and frequency of amino acid occurrence in epitopes. It is clear that improvements in prediction techniques are necessary and comparisons between predictions and empirical epitope mapping can contribute. The *in silico* predictions for epitopes in eqIgG3 revealed thirteen candidates according to the Kolaskar scale and twelve candidates by Bepipred. Some sequences were predicted by both methods, however, the Kolaskar scale (with a threshold set at 1.0) predicted longer sequences than Bepipred (with a threshold set at 0.35).

Two main criterias for consideration arose while analyzing potential candidate antigenic epitopes within eqIgG3. The first was the high sequence homology (60.3%) between rabbit and equine IgG3 heavy chains. Since antigens in rabbit heavy chains recognized by rabbit antibodies should be eliminated during the process of self tolerance, the epitopes in eqIgG3 that displayed homology to rabbit sequences were eliminated after alignment of the two heavy chain sequences [Fig-2].

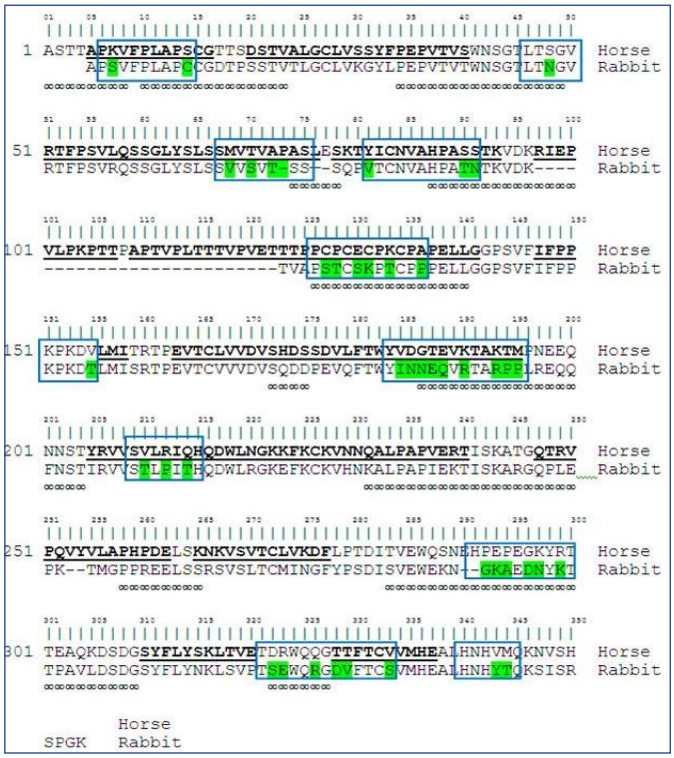


Fig. 2- Sequence alignment of the equine and rabbit IgG3 heavy chains and prediction for candidate epitopes. The sequence of eqIgG3 (access number AAA64252) is on top and for rabbit (access number K00752) on the bottom. Depicted in boxes are the epitopes determined by the experimental method of Spot-synthesis. Epitopes identified by the predictive methods of Kolaskar and Bepipred are marked under the sequences by "∞∞". Amino acids with greater than 30% exposure to solvent are in bold. Gaps are shown as dashed lines indicated. The alignment was performed at Clustal W. The overall identity was 60.3% and specific differences in amino acids within epitopes are highlighted in green.

Another criteria to satisfy from the epitope predictions within the IgG3 heavy chain was the requirement for surface residues to maintain accessibility for binding antibodies. IgG is a four chain protein and some residues are localized at the interface surface between chains such as between the CH1 domain and light chains or between the CH3 domains. To evaluate surface residues, a model of the eqIgG3 heavy chain was generated and the quality of the model evaluated using Prochek Ramakandran´s plot. Only one amino acid (S288) was located in the absolutely prohibited area of the plot (data not shown). After this analysis, eight candidate epitopes were eliminated for consideration to create targeted antibodies since they contain amino acids located in regions that contribute to the interfaces of the molecule. However, when the solvent exposure of amino acid chains was evaluated, three epitopes (eqIg7-YVDGTEVKTAKTM; eqIg9- HPEPEGKYR; eqIg11-HNHVMQ) were identified that, according to the programs, had a much higher probably to be involved in the interactions of antigen with antibody based on the presence of amino acids with greater than 30% exposure [Fig-2, amino acids in bold].

Antigenicity of the eqIg-7 Synthetic Peptide and Specificity of the Antisera

When comparing the performance of the three epitopes [Fig-1, middle], the most intense antigenic determinant (eqIg-7) was chosen for a creating targeted antibodies. For immunizing rabbits, this core sequence was expanded with two glycines at both the amino and carboxyl terminals along with a cysteine at the N-terminus (CGGTWYVDGTEVKTAKTMGG). The glycines functioned as spacers for improving interactions between antigen and antibodies. The cysteine was added to facilitate the conjugation of the peptide to tetanus toxoid (TT).

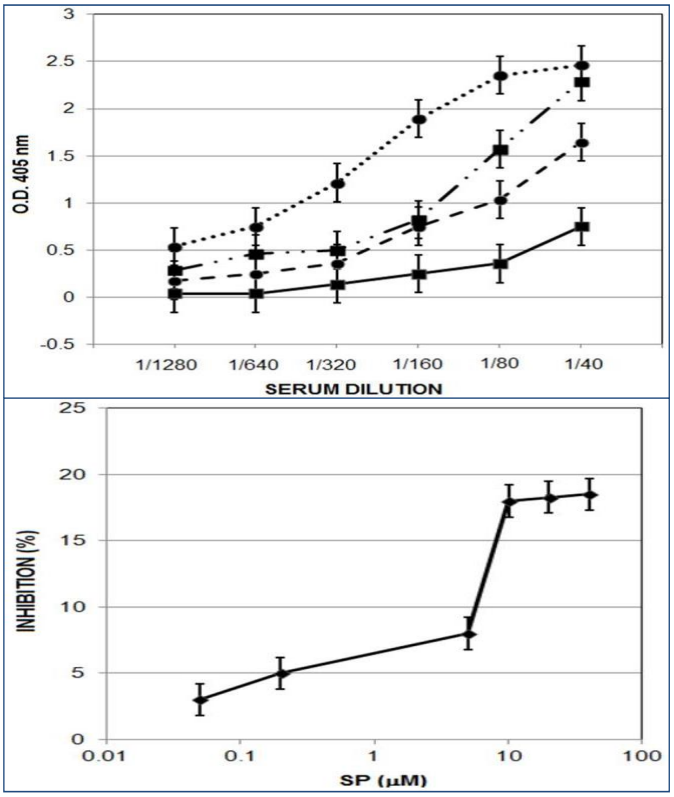


Fig. 3- Performance of sera from rabbits immunized against a synthetic peptide (SP). Panel A shows the reactivity of sera from two rabbits immunized with the SP (● Rabbit #1 and ■ Rabbit #2) from samples collected at 42 days (— and ▯ ▯ ▯) and 63 days (… and …▯ ▯...) after the first injection for purified eqIgG by direct ELISA. Panel B shows the competition analysis of rabbit anti-synthetic peptide-TT serum with purified peptide. Serum was incubated 1h at 37 °C with increasing concentrations of peptide (0.75 µM to 30 µM) before adding to hcIg-coated wells. Bound rabbit anti-SP antibodies were detected with a goat anti-rabbit Ig conjugated with HRP. Inhibition of binding (%) was calculated with 100% as the OD_{405} value in the absence of competing peptide.

Two rabbits were immunized independently with the eqIg-7-TT peptide and serum titers were monitored one week after the 3rd, 4th and 5th (final) immunizations [Fig-3A]. The titers against eqIgG3-7 specifically increased over time to almost the same extent according to the results from the direct ELISA, the titer for antisera against eqIgG3-7 increased greater than 32 times in the sera from each of the two rabbits comparing the start of the immunization to the end. Between the second booster (day 42) and the third inoculation (day 63), the titer increased 3.3 times. The specificity of antisera against eqIg-7-TT was analyzed by a competition experiment. As seen in [Fig-3B], the addition of free peptide eqIg-7 to antisera specifically inhibited the rabbit antibodies binding to eqIg adsorbed to ELISA plates. A significant level (20%) of inhibition was obtained at micromolar concentration.

Yet, the ability of antiserum anti-Ig to cross-react with various heterologous immunoglobulins is widely known and reflects the genetic similarities in this family of proteins across various species. We hypothesized that analyzing the amino acid sequences of candidate epitopes could improve the prediction for successfully creating targeted antibodies, which could minimize or eliminate the often observed cross reactivity. The alignments of the eqIgG3 with all of the other available immunoglobulin sequences deposited in data bank showed that the sequence of the eqIgG3-7 peptide was sufficiently unique to warrant testing for specificity (data not shown). Actual specificity was confirmed by a dot-blot assay against a panel of immunoglobulins from different animals (data not shown).

Homology Modeling

To confirm the expected utility of a targeted anti serum against the epitope eqIgG3-7, the 3-dimensional structure of equine IgG3 sequence (AJ312379) was predicted using I-Tasser [24,25] through the online server [Fig-4]. Default values were used with the submitted amino acid sequence to perform the calculations. The best model obtained in I-Tasser was the pdb structure of 1HZH chain H, which showed good identity (65.6%) and similarity (74.9%). The model calculated a good confidence (c-score of -0.39), which was used to estimate its quality. The TM-score, which is a value that correlates with RMSD, was 0.66±0.13 suggesting that the overall topology was correct, but the overall resolution from RMSD was only 7.4 ± 4.3Å. The side chain positions in the best model were corrected by an "exhaustive search" in SwissPDB Viewer (4) (http://www.expasy.org/spdbv), then the structure was minimized in GROMACS 4.5.3 package (5-8) using Charmm27 force field [26] and the steepest descent algorithm until convergence of 1000KJ.mol^{-1} on each atom resulting in RMSD of 0.11Å. Results were visualized with Visual Molecular Dynamics version 1.9 (VMD) [27].

Discussion

Preparations of equine sera are important therapeutically for the rapid and effective treatment of patients suffering from exposure to a variety of acute toxins. In a subset of people, there is a risk for life threatening hypersensitivity reactions, which has been principally attributed to immune-based allergic responses against whole equine immunoglobulins [9,28]. While cleavage of equine immunoglobulins into fragments diminishes the risks, preparations still often contain sufficient levels of whole eqIg to elicit hypersensitive reactions. The development of specific antibodies against intact eqIg proteins has been attempted to measure and eliminate these contaminants using several methodologies including the monoclonal antibodies technology, sera absorption and/or injecting peptides designed through *in silico* predictive modelling. Too often, the uniqueness of a peptide is the only criteria considered for peptide design and improvements are still needed.

Considering the methodology of spot synthesis as a resolute technique for identifying the exact amino acids that encompass the sequence of small epitopes, we applied this technique to eqIgG3 to define an epitope for generating a targeted antiserum that specifically and selectively bound intact eqIg. An initial consideration was that antibodies can mediate different effects and functions during an immune response depending on their class. Some can activate the classical pathway of the complement cascade when bound to antigen [29], others interact with Fcg receptors (FcgR) on various cells of the immune system, which mediate responses like phagocytosis, antibody-dependent cytotoxicity or immunoregulatory signaling of proliferation and antibody secretion. These interactions occur through the constant region domains (isotypes) of the antibody heavy chains [30,31].

Therefore, we investigated the fine structures of the linear antigenic determinants of the constant regions (CH1-CH3) in the heavy chain equine IgG3 protein. Based on the reactivity patterns of a commercially available hyper immune rabbit serum against a library of synthetic fifteen-peptides, eleven major antigenic determinants were identified. Four of these epitopes (eqIg-1-4) were localized in coil regions of the CH1 domain, precisely within the variable region of F(ab´)$_2$, one (eqIg-5) in the hinge, two (eqIg-6-7) in the region of constant domain CH2 and four in the region of constant domain CH3 (eqIg-8-11).

The epitopes discovered by the other two predictive methods, [Fig-2], suggested 12-13 epitope candidates, close to the number of epitopes identified by SPOT synthesis. Only one epitope (eqIg5) was relatively superimposed by all three approaches. In addition, the predictive methods tended to select longer stretches of amino acid for antigenicity then the SPOT-based analysis and, in some instances, within regions not accessible for antibody binding. These results confirmed previous observations that the quality of predictions for B cell-epitopes often of low quality for use as a reliable tool by immunologists [32,33]. In contrast, the predictions used to identify T-cell epitopes appear to be more accurate [34,35].

While the use of sequence alignments and molecular modelling improve the results from predictions by eliminating candidate epitopes, it is still impossible to determine if the presence of an individual amino acid is critical for inducing antibody production or antibody recognition and binding. A powerful aspect of using synthetic peptides as antigenic probes is their ability to ascertain the essential amino acids in an antigenic sequence. It has been demonstrated that most of the linear peptide epitopes have between two to six critical residues, usually located within a sequence flanked by seven to ten residues in length [36,37]. In the case of the eqIg-2 and eqIg-6 epitopes, which were recognized antigens for rabbit anti-equine sera, there is only one amino acid that distinguishes the equine sequence from the corresponding orthologous rabbit sequence [Fig-2]. This strongly demonstrates that a single residue can define the antigenicity of an epitope for antibody recognition and binding.

From the 11 epitopes identified by SPOT analysis, the eqIg-7 demonstrated the greatest reactivity [Fig-1]. It contained the most amino acids with a high solvent exposure, a greater divergence between equine and rabbit sequences and two loops, the first one in G^{186} and the second 190K-A192. This epitope was not predicted by Kolaskar Tongaonkar scale, perhaps because this scale does not use secondary structure as a parameter. The epitope eqIg-9 had some features in common with the eqIg-7, especially a large divergence from the rabbit sequence. This region has deletion/insertion of two amino acids and two loops in its structure, 290N-P292, and G296. The first loop is complete and was more reactive. However, one major difference was that the sequence of the eqIg-7 epitope is more conserved with the other classes of equine immunoglobulins, with most of its amino acids identical to those in six of the seven isotypes of IgG suggesting that antibodies against this epitope should recognize all isotypes of IgG.

The most surprising result was with the eqIg-11 epitope. This epitope showed an average reactivity and also was not predicted by

either of the two programs. This epitope forms a loop between residues 339H-V343 [Fig-4]. Spot 20°C, where the loop is complete, showed moderate signal intensity. In the alignment of equine and rabbit immunoglobulins, the epitope eqIg-11 showed that the main difference between the rabbit and the equine are the residues 343V -M344. These amino acids are hydrophobic by the scales of Kyte-Doolittle and Parker and could explain why it was not identified by the predictive programs from the primary structure. Despite the importance of polar sequences for the formation of antigen-antibody complex, the individual polar residues can also be important for complex formation. Similar results were highlighted previously with the demonstration of the importance of leucine residues in a cyto-megalovirus virus epitope [19]. Another important feature of this epitope is the presence of the dipeptide 343V-M344 which could confer IgG3 isotype specificity to the eqIg11.

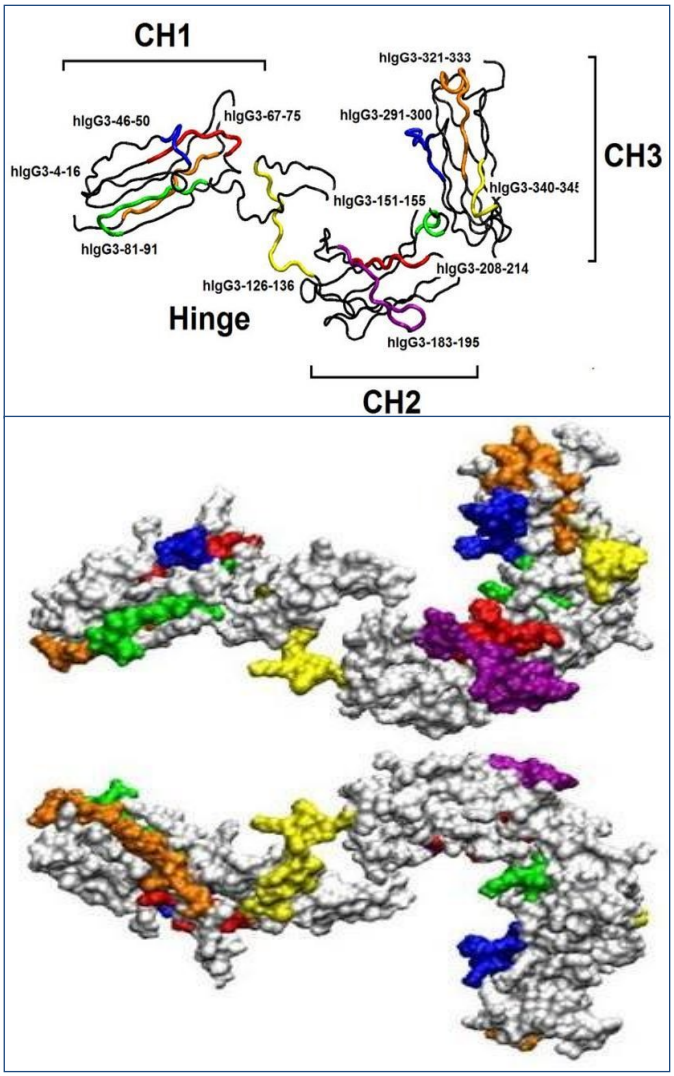


Fig. 4- Spatial localization of the B-epitopes on the predicted 3D-structures of eqIgG3 heavy chain (access number aj312379). On the left, a ribbon diagram shows antibody domains and the identified epitopes in color. On the right, a space filling representation displaying the surface localization of the identified epitopes. Models were obtained through the I-Tasser server and viewed by VMD.

Yet, the eqIg-7 epitope presented the highest reactivity and had a structural divergence that prompted its selection for validation studies. The results presented in the [Fig-3] show that a monospecific antisera was produced in rabbits through immunization with the peptide coupled to tetanus toxoid. The performance of the antisera against eqIgG3 a new and innovative method for detecting intact IgG in the presence of equine F(ab') 2 fragments. It should be useful as a reagent for quality control tests to identify and quantify whole eqIg within industrial preparations of equine immunotherapeutic biotechnological products used antivenoms. Another possible application of this type of targeted antibody production is its use in affinity chromatography for purification and elimination of IgG3 from hyperimmune plasma.

Conclusion

The data presented in this paper describes for the first time an effective approach to study linear epitopes in the heavy chain of eqIgG3 protein. This approach should provide a useful method to probe the fine structure of various amino acid sequences for biotechnological applications either for both human or veterinarian uses. The framework of considerations applied to the design of a peptide allowed the development of specific antibodies for the IgG3 subclass. The preparation of antibodies specific for equine IgG3 through the use of synthetic peptides may help obtain better results in diverse immunological detection. This simple approach may serve to prepare specific antibodies for other purposes as well.

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