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Generation And Preliminary Characterization of Murine IgM Monoclonal Antibodies against the N-terminal β-strand Of A Fibrillogenic Light Chain Variable Domain

Olga Mata Ruiz[1], Yolanda Medina Flores[1*], Miying Dessire Gómez Cervantes[1], Diana Reynada Castillo[1], Robin Axel Ruiz Zamora[2], Francisco Javier Rodríguez Álvarez[2], Karen Cortes Sarabia[1], Luis del Pozo Yauner[2].**

[1] Lab. De Anticuerpos Monoclonales, Instituto de Diagnóstico de Referencia Epidemiológica, México D.F., México.

[2] Lab. De Estructure de Proteína, Instituto Nacional de Medicina Genómica, México D.F., México.

Abstract: Based on findings of previous studies, we have identified the N-terminal segment of the light chains variable domains as a structurally important region for the mechanism of amyloid fibril assembling. Thus, we have suggested that this region could be target for monoclonal antibodies (MAb) with the potential for inhibiting the amyloid aggregation of the light chains, and/or suitable for the inmunohistological detection of the AL deposits. In this work, we report the generation and preliminary characterization of a group of murine monoclonal antibodies against the N-terminal β strand of the fibrillogenic variable domain protein 6aJL2. The immunization of the laboratory animals (three female Balb/c mice 4-6 weeks old) was performed by intraperitoneal inoculation of a mix composed by the synthetic peptide Asn1-Lys17 (50 μg per animal) homogenized with the adjuvant TiterMax® Gold (Sigma-Aldrich Cat No. T2684). The generation of the hybridomas was accomplished by the conventional methodology, using murine myeloma cells X63Ag8.653. The hybridoma screening and antibody characterization were performed with two different methodologies: indirect ELISA and dot-blot. In both techniques, the antibody recognition was evaluated against the synthetic peptide Asn1-Lys17 and the recombinant protein 6aJL2, from which the peptide originated. Ten different hybridomes were selected by the capacity to produce monoclonal antibodies (MAb) that bind the peptide Asn1-Lys17. Interestingly, all the MAb

were of IgM class, as determined by indirect ELISA. The preliminary characterization indicates that all the antibodies recognize the 6aJL2 protein in the fibrillar state, but not in the native conformation.

Key words: Monoclonal antibody, hybridome, amyloid fibril, 6aJL2 protein, light chain.

INTRODUCTION

Primary systemic amyloidosis, also known as AL amyloidosis, is caused by the systemic deposition, in the extracellular compartment, of a monoclonal free light chain in the form of insoluble fibrillar aggregates[1]. Although the molecular mechanism of the light chain self-assembling into amyloid fibrils remain being an unresolved issue, more than three decades of researches have led to the identification of a group of factors driving the amyloidogenic behavior of this family of immunoglobulin[2]. As it seems to be true for almost all the amyloid precursor proteins, the evidence indicates that the thermodynamic stability of folding is one of the major factors modulating the amyloid-forming potential of the light chains. It has been consistently observed an inverse correlation between the thermodynamic stability of the light chain and the propensity to aggregate as amyloid fibrils in *in vitro* studies[2-5]. Therefore, the kinetic of AL fibril formation can be accelerated by destabilizing the native state of the light chain by means of point mutations at structurally relevant positions[3,6-8]. Similar effect can be achieved by incubating the protein in destabilizing conditions, as low pH, high temperature or middle concentration of the denaturing substances urea and guanidine hydrochloride[9-11]. In conjunction, these findings give support to the misfolding hypothesis of the light chains amyloidogenesis, which postulates that at least part of the light chain adopts a non-native conformation in the fibrillar state and that such conformational rearrangement is a key component of the mechanism of self-assembling of the protein into amyloid fibril[3,5,11,12]. It is believed that the misfolding causes that some aggregation promoting regions, normally buried in the native core, get access to the molecular surface, where they are available for intermolecular promiscuous contacts leading to aggregation[13,14]. Thus, the misfolding hypothesis implies that the amyloidogenesis can be avoided by stabilizing the native state and/or by hindering the intermolecular contacts by binding the aggregation promoting sequences with specific ligands [15-17]. The antibodies have proven to be capable to perform both actions, being recognized since a long time as a promising option for developing anti-amyloid agents[18]. In a previous study, we have obtained evidences suggesting that the N-terminal segment, comprising the β strand A, modulates the amyloidogenesis of the variable domain of the light chain (Figure 1)[19]. It has been shown that mutations that disrupt structurally relevant interactions for the N-terminal segment promote the aggregation of the recombinant variable domain 6aJL2, shortening the lag time (t_{lag}) of the fibrillogenesis[7,19]. In some cases, the effect seems to be better explained by the destabilization of the domain folding. However, we also obtained evidences suggesting that some mutations at the N-terminal segment influence the mechanism of aggregation in a sequence-dependent manner, irrespective of their impact on the folding stability[19]. Based on these findings, we have anticipated that the N-terminal segment could be involved in contacts relevant for the mechanism of fibril assembling, contributing in some extend to stabilizing the core of the fibrils. In line with this suggestion, we found that the N-terminal segment is buried in the fibrillar state[19]. Thus, we proposed that the N-terminal segment of the light chains variable domains could be an appropriate target for monoclonal antibodies (MAb) with the potential for inhibiting the amyloid aggregation, and/or suitable for the immunohistochemical typing of the AL deposits.

This work was aimed to the generation and preliminary characterization of a group of murine MAb that were generated by immunizing Balb/c mice with the synthetic peptide Asn1-Lys17 (NFMLTQPHSVSESPGK), which has the sequence of the first sixteen residues of the N-terminal segment of the 6aJL2 protein.

METHODS

Recombinant protein expression and purification: The 6aJL2 is a germline-encoded recombinant light chain variable domain (rV_L) protein belonging to the subgroup λ6. The expression and purification of this molecule were performed as described previously[6].

***In vitro* fibrillogenesis of the 6aJL2 protein:** A 500 µg/ml dilution of 6aJL2 protein in PBS buffer pH 7.4 was incubated at 37 ºC with constant orbital stirring (1000 r.m.p.) in a 2 ml capped plastic tube (Eppendorff). At predetermined time, an aliquot (25 µl) was withdrawn from the sample and the presence of amyloid aggregates was determined by the Thioflavin T assay[20]. The aggregates of the endpoint sample were harvested by centrifugation, washed twist with one volume of PBS buffer pH 7.4 and resuspended in the same buffer. The concentration of the amyloid fibril suspension was determined spectrophotometrically at 280 nmn in 5.5 M guanidine hydrochloride solution, as described previously[7].

Polyclonal antisera: Rabbit polyclonal antiserum against the N-terminal segment of 6aJL2 protein was purchased from GenScript USA Inc. (Piscataway, NJ, USA). The antiserum was produced by immunizing the lab animal with peptide Ans1-Ser12 (FMLTQPHSVSC), covalently linked to a protein carrier by means of a Cys residue inserted at the C terminal position of the peptide.

Production of monoclonal antibodies: Balb/c female mice (6-8 weeks old) were injected with 100 µl of a mixture containing 50 µg of the synthetic peptide Asn1-Lys17 (GenScript USA Inc), homogenized in adjuvant TiterMax® Gold (Sigma-Aldrich Cat No. T2684). The injections were repeated four more times with equivalent doses at 10-day intervals. Following the final boost, mice were rested for 5 weeks and then injected with 50 µg of peptide Asn1-Lys17 homogenized in adjuvant TiterMax® Gold. Four days later, the spleen cells were removed and fused with X63Ag8.653 (ATTC: CRL 1580) myeloma cells using 50% polyethylene-glycol 4000 as a fusion agent according to the standard techniques (Sigma-Aldrich). Hybridoma cells were grown in hypoxanthine-aminopterin-thymidine (HAT) selection medium containing 15% fetal bovine serum on wells of 96-well microplates (Maxisorp nunclon, Denmark), under humidified conditions at 37 ^{0}C with 5% CO_2. After 10 days, the supernatants of the cultures were tested by ELISA to identify wells that contained cells that produced anti-Asn1-Lys17 peptide antibodies. Cells in wells that tested positive were frozen in liquid N_2 and were later thawed to be cloned. Some wells were chosen for amplification and cloning based on the strong immunoreactivity of their cell supernatant, and the cells were cloned at least twice by limiting dilution. Clones secreting antibody of desired reactivity were expanded *in vitro*, they were grown at density in 75 mL flasks (Nunclon, Denmark). Antibodies were purified by HiTrap IgM HP chromatography (Bio-Rad Laboratories). Isotype was determined using a peroxidase labeling immunoassay kit (Bio-Sciences Laboratories) according to the procedure recommended by the manufacturer.

Indirect Enzyme-Linked Immunosorbent Assay (ELISA): The screening of mAbs to antigens was performed by an indirect ELISA as follows: To each well of a 96-well plate (Maxisorb Nunclon, Intermed, Denmark) was added 100 µL of Carbonate Buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.2 g/L NaN_3, pH 9.6) containing the synthetic peptide Asn1-Lys17 5 ug/mL and allowed to bind overnight at 4°C. Following the removal of the binding solution, the plates were rinsed three times with PBS-Tween 20 0.3 %, and 5%

skimmed milk (blocking buffer) to saturate non-specific protein binding sites and then incubated for 30 min at room temperature.

The solution was shaken out, and the plates were rinsed in PBS-Tween 20 0.05 %, and drained. At this time, 100 µL of culture supernatant was added to the wells, and the plates were incubated at 37^0 C for 2 h. The plates were then washed three times with PBS-Tween 20 0.3 %, and 100 µL of a 1:8,000 dilution of a peroxidase-conjugated Goat anti-mouse IgM polyclonal antiserum (Jackson ImmunoResearch, West Grove, PA, USA) was added to each well. After incubation for 2 h at 37^0 C , the plates were washed three time with PBS-Tween 20 0.3 % and then, 100 µL per well of a solution containing ortophenylendiamine (0.4 mg/mL) and 1% H_2O_2 in citrate buffer pH 5.5 was added.

The plates were incubated at 37^0 C and the absorbance of each well was determined at 492 nm in a Tecan Sunrise™ Microplate Absorbance Reader (Tecan Group Ltd., Männedorf. Switzerland).

Dot-Blot Immunoassay: An aliquot (2 µl) of each sample (166 µg/ml) was spotted in duplicate on a Hybond PVDF membrane strip (GE Healthcare Life Science) and left to dry for 45 min. Nonspecific sites were blocked for 2 hours in a 5% w/v dilution of Svelty skimmed milk in PBS Buffer pH 7.4 plus 0.05% tween 20. The membrane was incubated overnight with a proper dilution of the primary antibody in PBS buffer pH 7.4 plus 0.05% tween 20 and then it was washed several time with PBS buffer pH 7.4 plus 0.05% tween 20 (washing buffer).

The membrane was incubated with a horseradish peroxidase conjugated secondary antibody diluted 1:2000 v/v in washing buffer for one hour and then washed several time as previously described. The antigen-antibody reaction was revealed by incubating the membrane with a proper volume of Immobilon Western Chemiluminescent HRP Substrate (Merck-Millipore, Merck KGaA of Darmstadt, Germany) for one minute. The excess of solution was removed from the surface of the strip and the chemiluminiscence registered in a Molecular Imager VersaDoc MP imaging systems (80 seconds total exposure, with 20 seconds between captures).

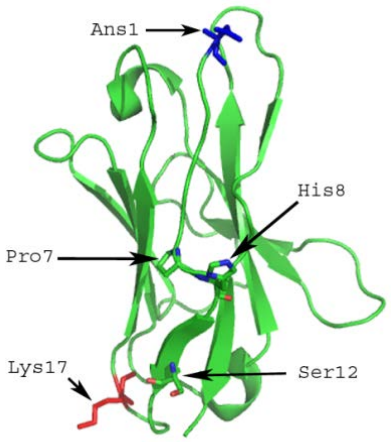

Figure 1: Structural representation, in cartoon format, of the recombinant variable domain protein 6aJL2, determined by x-ray crystallography (PDB I.D. 2W0K) (7). Residues Asn1, Pro7, His8, Ser12 and Lys7 are represented in stick format. The image was generated by PyMOL software.

RESULTS AND DISCUSSION

1. **Hybridoma screening and isotype determination:** As is described in Methods, the screening of the hybridomas generated in the cell fusion step was performed by means of an indirect ELISA assay. Three different molecules; the synthetic peptide Asn1-Lys17 in two structural formats, as a free molecule and in the form of multi-antigenic peptide (MAP), and the protein 6aJL2 in the native state; were used as antigens to evaluate the recognition properties of the antibodies present in the culture supernatants. Ten hybridomas secreting antibodies with the capacity to recognize the peptide Asn1-Lys 17 were identified (Figure 2 panel A). The absorbance at 495 nm, which reflects the amount of antibody molecules bound to the antigen, varied from one sample to another, suggesting differences in the antibody concentration, or regarding the strength of the antigen-antibody interaction, or both (Figure 2 panel A). As a tendency, the signal for the recognition of the peptide Asn1-Lys17 in MAP format was higher than for the free peptide, which is probably explained by a better availability of the molecular epitopes in the MAP structure[21]. The indirect ELISA used through the hybridoma screening was based on an affinity-purified peroxidase-conjugated IgG (H+L) specific antiserum (Jackson Immune Research). Thus, the capacity of the peroxidase-conjugated antiserum to recognize both the γ heavy chain and κ light chain of the antibodies allowed the selection of hybridomas secreting Asn1-Lys17 peptide-recognizing antibodies belonging to classes other than IgG. We used a second peroxidase-based immunoassay for accurately determining the antibody isotype and it was found that the ten antibodies with peptide binding capacity are of IgM class (Figure 2 panel B). This was an interesting but unexpected finding that strongly suggests that the immune humoral response elicited by immunizing of Balb/c mice with the peptide Asn1-Lys17 mixed with the adjuvant TiterMax® Gold relied mainly in IgM-producing B-cells. It has been reported that the immunization of non-autoimmune Balb/c mice with a peptide mimotope of DNA elicited an autoantibody response formed by a fraction of IgM antibodies. It was shown that such IgM autoantibodies harbored somatic mutations, indicating that they were generated as part of the antigen-driven response[22]. Long-lived IgM-expressing B cells can be induced in mice upon exposure to T cell-independent Ags[23]. Similarly, most of the human memory B cells recognizing the antigens Rh and tetanus toxoid reside in IgM+ B cells; it was also shown that the IgM antibodies that they produce harbor somatic mutations[24]. At the present stage of this research, there is not sequence data available. Thus, it is not possible to perform a more extensive analysis regarding the structural properties of our anti-peptide IgM antibodies.

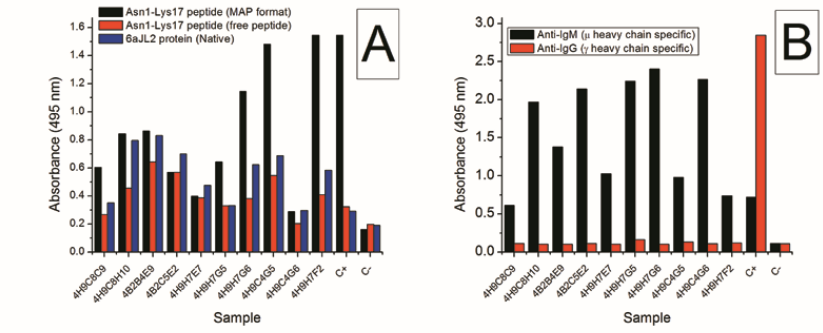

Figure 2: Enzyme-linked immunosorbent assays (ELISA) performed for: A) determining the recognition properties and B) to identify of the heavy chain isotype of the antibodies secreted by the hybridomas.

2. **Recognition properties of the anti-peptide IgM MAbs:** To go deeply into the characterization of the recognition properties of the IgM MAbs, we performed a dot-blot immunoassay for testing their capacity to recognize the protein 6aJL2 in two different states, as soluble native molecule and in the form of amyloid-like fibrillar aggregates. Interestingly, all the IgM MAbs recognized the amyloid-like fibrils of 6aJL2 protein, but not its native state (Fig. 3). Such result contrasts with the data obtained by the indirect ELISA immunoassay, where a variable degree of recognition of the native state of 6aJL2 protein was observed (Fig. 2). Two additional reagents, the MAb 55-5-F5 and a rabbit polyclonal antisera produced by immunizing the animals with the peptide Asn1-Ser12, were tested as controls. Mab 55-5-F5 recognized the 6aJL2 protein in the two states, native and fibrillar, in contrast to the rabbit antiserum which recognized only the 6aJL2 fibrils, as did the IgM Mabs (Fig. 2). The observed differences in recognition capability of those antibodies could be explained, at least in part, by the structural differences between the molecules that were used for immunizing the lab animals in each case. The Mab 55-5-F5, kindly supplied by Dr. Alan Solomon and Dr. Jonathan Wall (The University of Tennessee Medical Center at Knoxville, TN, USA), is a λ6 variable region subgroup-specific IgG that was generated by immunizing mice with heat-precipitated human Bence Jones protein emulsified in monophosphoryl lipid A and trehalose dimycolate[25,26]. In contrast, the rabbit polyclonal antibodies as well as the IgM Mabs were generated by immunizing with peptides that are structurally very similar (see Methods).

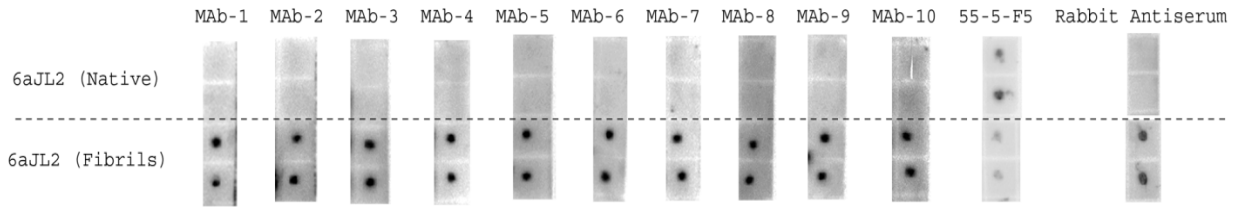

Figure 3: Dot-blot immunoassay performed for determining the capacity of the antibodies to recognize the 6aJL2 protein in two different conformational states, native and fibrillar. The reagents that were tested in this experiment are ten IgM Mabs (arranged in the same order as in figure 2, panels A and B), the polyclonal antiserum produced by immunizing rabbits with the synthetic peptide Asn1-Ser12 (Rabbit Antiserum) (purchased to GenScript Inc.), and the IgG MAb 55-5-F5.

The amyloidogenesis of protein and peptides is a nucleation-dependent phenomenon in which many different species participate[27]. In order to establish the capacity of some of the IgM MAbs to recognize those species, a dot-blot assay was performed in which samples taken at different time of incubation of 6aJL2 protein were tested. As it is shown in fig. 3A, both the MAb IgM-1 (4H9C8C9) and the rabbit polyclonal antiserum recognized only the aggregates formed after more than 5 hours of incubation, period of time that corresponds to the plateau phase of the fibrillogenesis kinetic of 6aJL2, characterized by a constant value of the ThT fluorescence (Fig. 3B). Again, the MAb 55-5-F5 recognized the 6aJL2 protein in both conformational states, native and fibrillar. Typically, the kinetics of fibrillogenesis of most proteins and peptides can be divided into three phases. The first phase, named *lag time* (t_{lag}), is characterized by a very low value of the signal used to detect the formation of amyloid fibrils, that in most cases is the ThT fluorescence. According to the nucleation-dependent model of light chain amyloidogenesis, during the t_{lag} the sample is populated by a diversity of oligomeric species, which are precursors of the stable nuclei[27]. After

stable nuclei are formed, the soluble monomers incorporate sequentially into the growing polymeric structure of the fibrillar aggregates[28]. The fast and continuous formation of fibrillar aggregates correlates with an exponential increase of the ThT fluorescence (Fig. 3B). Atomic Force Microscopy (AFM) analysis has revealed that the kinetics of formation and assembling of the light chain amyloid fibrils is a hierarchical process, that start with the formation of the single filaments[29].

The filaments, which are the predominant aggregates at the early time of the exponential phase, combine to form the protofibrils, which are predominant at intermediate time. Finally, the protofibrils intertwine to form the mature fibrils, which are predominant at end-point samples[29]. Thus, our data suggest that, in the conditions established in our dot-blot immunoassay, the IgM Mab-1 and the rabbit polyclonal antiserum recognize epitopes that are expressed exclusively in the mature fibrils, but not in their precursors, neither in the native monomers.

In this regards, it was previously reported that the IgG1 MAb 11-1F4, generated by immunizing mice with a thermally denatured V_L of the human κ4 light chain Len, reacts with a non-native conformational epitope that is contained within the first 18 residues (N-terminal) of the protein.

It was shown that the MAb 11-1F4 epitope is characteristic of the partially denatured and fibrillar light chains, irrespective of the belonging to the kappa or lambda isotypes, but is not present in the native proteins [30,31]. Further studies, based on different experimental approaches, are needed for confirming the apparent specificity of binding to the mature fibrils of our IgM Mabs.

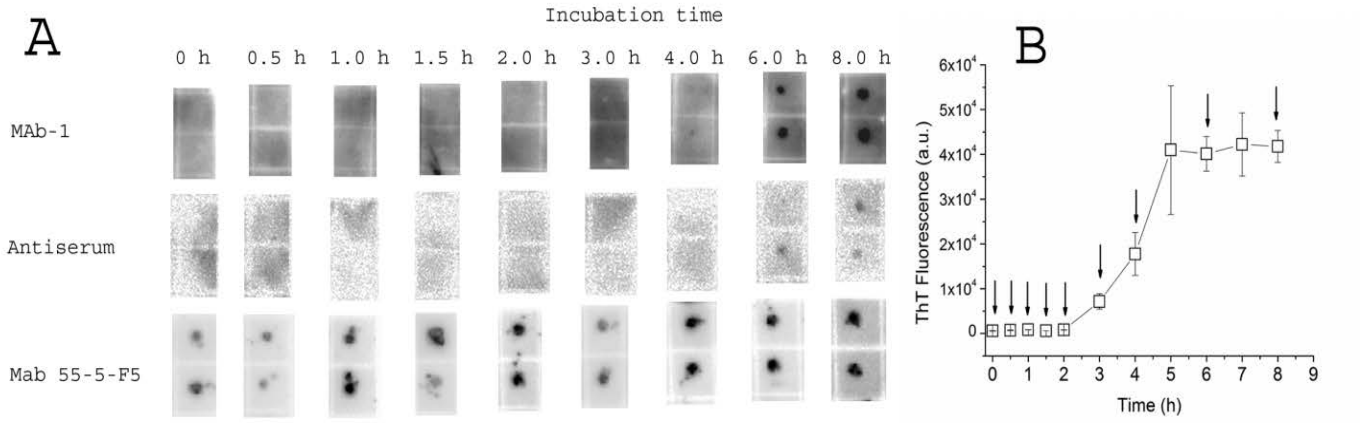

Figure 4: A) Dot-blot immunoassay performed for determining the capacity of the antibodies to recognize the components of samples taken at different times of incubation of the 6aJL2 protein. The reagents that were tested are IgM Mab 4H9C8C9 (AcM-1), the polyclonal antiserum produced by immunizing rabbits with the synthetic peptide Asn1-Ser12 (Antiserum) (purchased to GenScript Inc.), and the IgG MAb 55-5-F5. B) Kinetic of fibrillogenesis of the 6aJL2 protein, determined by ThT fluorescence (see Methods). The downward arrows indicate the times at which the samples tested in A) were taken.

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*** Corresponding authors: Yolanda Medina Flores and Luis del Pozo Yauner**

Lab. De Anticuerpos Monoclonales, Instituto de Diagnóstico de Referencia Epidemiológica, México D.F., México.
medinafy@yahoo.com.mx, ldelpozo@inmegen.gob.mx