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Murine Monoclonal Antibodies against the Antimicrobial Peptide Oreoch-2 from Tilapia (*Oreochromis niloticus*)

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***Abstract*—** Classic hybridoma technology has developed monoclonal antibodies (mAb) against a wide variety of antigens with several applications in biotechnology. In this work, murine mAb secreting hybridomas were generated for specific recognition of antimicrobial peptide Oreoch-2, isolated from tilapia and with demonstrated antimicrobial activity against bacteria and fungi. BALB/c mice were immunized with Oreoch-2 conjugated to bovine albumin. Cell fusion was performed between lymphocytes isolated from spleen of mouse with highest antibody serum titers and P3/x63.Ag8.653 myeloma cells. Three hybridomas with stable secretion of anti-P22 mAb were obtained, which did not show cross-reactivity with Oreoch-1 related peptide. The mAbs will be used in the study of the action mechanisms of antimicrobial peptide Oreoch-2 and to establish analytical tools for its detection and quantification in pharmaceutical formulations.

Keywords— ascitic fluid, cell fusion, ELISA, hybridoma

I. INTRODUCTION

Monoclonal antibodies (mAbs) are specialized glycoproteins of the immune system. The hybridoma generation theory developed by Köhler and Milstein in 1975 allowed obtaining mAbs capable of recognizing a single epitope and producing them in an unlimited way. Every year increase significantly the reports of new mAbs obtained against an immense variety of antigens, which supports the extraordinary importance of this technology and its infinite application in the current development of biotechnological sciences [1].

Antimicrobial peptides have also opened a wide field of research in biotechnology. They are a crucial part of the innate immunity that exists in most living organisms [2]. Antimicrobial peptides are formed by short amino acid chains with hydrophobic characteristics and a positive charge, which alter the lipid bilayer of microorganisms, causing loss of ions and metabolic substances until they cause death. In this sense, antimicrobial peptides are considered natural antibiotics present in all forms of life, from unicellular organisms to mammals, and they are not affected by mutations that cause resistance to classic antibiotics [3].

In 2013, three antimicrobial peptides called Oreochromycin (Oreoch-1, Oreoch-2 and Oreoch-3) were isolated from tilapia (*O. niloticus*) [4]. One year later, it was demonstrated that Oreoch-1 and -2 have a broad spectrum of antimicrobial activity against bacteria and fungi, in addition to improving the innate immune response in BALB/c mice and in tilapia [5]. These results provide important insights for applying these peptides as molecular adjuvants and for the treatment and/or prevention of microbial diseases in fish and mammals [6].

The aim of this work was the generation of mouse mAb secreting hybridomas that specifically recognize the antimicrobial peptide Oreoch-2, as well as the *in vivo* production and purification of mAb. These mAbs would be useful to study the mechanisms of action of the antimicrobial peptide Oreoch-2. It could also be used to establish analytical tools that allow the detection and quantification of the peptide in pharmaceutical formulations that need to be developed to use it as new generation antimicrobials.

II. MATERIALS AND METHODS

Antigens: Antimicrobial peptide Oreoch-2 (P22) with 25 amino-acids and 2.97 kDa; antimicrobial peptide Oreoch-2 conjugated to bovine serum albumin (P22-BSA); antimicrobial peptide Oreoch-1 (P21) with 23 amino-acids and 2.52 kDa.

Animals: Five seven-week-old female BALB/c mice, with a weight between 20 and 25 g, were supplied by the National Center for the Production of Laboratory Animals (CENPALAB), Havana, Cuba.

Immunization schedule: Before starting the immunization schedule, blood samples were collected from each mouse to be used as preimmunized control serum. The mice

received three subcutaneous immunizations with intervals of 28 days. The first dose was 100 µg of P22-BSA emulsified in Freund's complete adjuvant. The second and third immunizations were administered with 50 µg of P22-BSA in incomplete Freund's adjuvant. Ten days after the last injection, blood was obtained for serum titration of specific antibodies by ELISA. Three days before fusion, the mouse with highest serum titer was inoculated intraperitoneally with a 50 µg booster dose of P22-BSA dissolved in phosphate buffered saline (PBS).

Indirect ELISA for the titration of IgG anti-P22 antibodies in sera from immunized mice: Costar® 3590 high binding plates were coated with 100 µL well of 10 µg/mL of p22-BSA diluted in 10 mM carbonate-bicarbonate buffer (pH 9.6) and incubated 2h at 37°C. After three washes with PBS-0.05% Tween 20 (PBST), the wells were blocked with a solution of 3% nonfat milk in PBST for 1h at 37°C. The wells were washed once with PBST. After that, 100 µL of the sera of the immunized animals, diluted with PBST and 1% nonfat milk in serial dilutions 1:2 (from 1:1000 to 1:128000) were added to the wells. A mixture of preimmunized sera was used as a negative control. The samples were incubated 2h at 37°C. After three washes with PBST, the recognition step of anti-P22 antibody bound to the antigen on the plate was performed, using an anti-mouse IgG-horseradish peroxidase conjugate (Sigma, USA), diluted 1:10000 in PBST and 1% nonfat milk, 1h at 37ºC. After four washes, the enzyme-substrate reaction occurred with 100 µL/well of 0.6 mg/mL ortho-phenylenediamine in citrate buffer (pH 5.0) with 0.015% H_2O_2, for 30 min in the dark at (22-25) °C. The reaction was stopped with 100 µL/well of 2.66 M HCl. The absorbance was measured by a plate reader (Labsystems Multiskan® Plus, Finland) at 492 nm.

Cell fusion, detection and cloning of antibody-secreting hybridomas: Spleen cells from mouse with higher antibody titers and exponentially growing mouse myeloma cells of the P3/x63.Ag8.653 line, were used to perform cell fusion with 50% (w/v) polyethylene glycol solution (Sigma, Hybri-Max) according to the method of Kohler and Milstein [7], to obtain hybridomas that were distributed in 96-well culture plates, resuspended in RPMI-HAT selection medium (Sigma, Hybri-Max) with 20% of fetal bovine serum. The plates were incubated at 37°C in a 5% CO_2 atmosphere. After 10 days of culture, hybridomas with the capacity to produce specific mAbs against P22 were detected by the ELISA described above, using the undiluted culture supernatants as samples. Mouse serum with higher titers of anti-P22 antibodies was used as a positive control for this assay, and mAb-secreting hybridoma supernatants against unrelated antigens were used as a negative control. The same ELISA protocol was used to evaluate the possible reactivity of the antibodies against P21 (Oreoch-1) as a related peptide, as well as the recognition of unconjugated P22 to bovine serum albumin (BSA). For this, three independent ELISAs were performed by coating the plates with these antigens. The results were expressed as the mean of absorbance at 492 nm.

Determination of the mAb isotypes: The classes (IgM, IgA or IgG) and the IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) of the antibodies present in the supernatant of the positive hybridomas were determined. Identification was carried out using a mouse monoclonal antibody isotyping kit (Sigma, Germany), according to the manufacturer's recommendations.

Production of mAbs in ascites and purification: The cells of the selected hybridoma were washed 2 times in RPMI-1640 medium and centrifuged at 180 g for 10 min at (22-25) °C. These cells were resuspended in RPMI-1640, to obtain a concentration of $1x10^6$ cells per milliliter of medium, which was inoculated intraperitoneally to 20 BALB/c mice, 1 mL per mouse. The peritoneum of the mice was stimulated with paraffinic mineral oil ten days before administration. Seven days after the inoculation, the ascites fluid was extracted by puncture of the peritoneum and clarified by centrifugation at 1125 g for 30 min at (22-25) ºC. The ascites fluid were filtered through glass wool 0.45µm and precipitated with 50% (w/v) of ammonium sulfate. After 30 min of centrifugation at 180 g, the pellet was resuspended in equilibrium buffer (1.5M glycine, 3M NaCl, pH 8.9) and loaded onto an nProtein A Sepharose Fast Flow matrix (GE Healthcare) packed in a C10/10 column (GE Healthcare) with a height of 8.5 cm. Elution was performed with 0.1M citrate buffer, pH 6.0. The flow was kept at 0.5 ml/min throughout the process. The chromatographic profile was monitored by measuring the absorbance at 280 nm. The eluate was neutralized with 2M Tris-HCl and dialyzed in 20 mM Tris-HCl, 150 mM NaCl, pH 7.0, during 16 h, and then filtered through 0.2 µm membranes. Thimerosal was added as a preservative to a final concentration of 0.02% (v/v). Purification checking was performed with an electrophoresis on a denaturing 12.5% polyacrylamide gel with 2% Sodium Dodecyl Sulfate (SDS). Purified anti-P22 mAb and anti-IL2h mAb (used as positive control) were diluted in loading buffer (125 mM Tris-HCl pH 6.8, 1% SDS, 5% glycerol, 10 mM dithiothreitol, 0.005% bromophenol blue) and boiled for 10 min. Both samples were put into the gel and they run for 45 min at 200 V in a buffer with 25 mM Tris-HCl pH 8.8, 192 mM glycine and 35 mM SDS. The gel was stained for 20 minutes with 0.1% Coomassie R-250 solution, 40% methanol, 10% acetic acid. The destaining of the gel was performed with 10% methanol and 10% acetic acid, until the visualization of the bands.

III. RESULTS AND DISCUSSION

All the immunized mice reached antibody titers in serum, with absorbance values between 0.569 and 3.444 in the dilution range between 1:1000 and 1:16000 (Fig. 1). In all cases, the IgG titers were higher than those obtained in preimmunized sera. Mouse 3 presented the highest antibody titers in serum and it was selected as a source of splenocytes for the fusion with myeloma cells.

By fusing immortalized myeloma cells with mouse splenocytes, all produced hybridomas can secrete mAbs

against the entire repertoire that constitutes the immune response of the experimental animal [9]. In this work, as a result of cell fusion, from 864 seeded wells, 311 wells were obtained with growth of the fused cells, which survived the selection with the HAT medium, for a fusion efficiency of 36%. Fusion efficiency values between 35 and 60% are considered satisfactory [10].

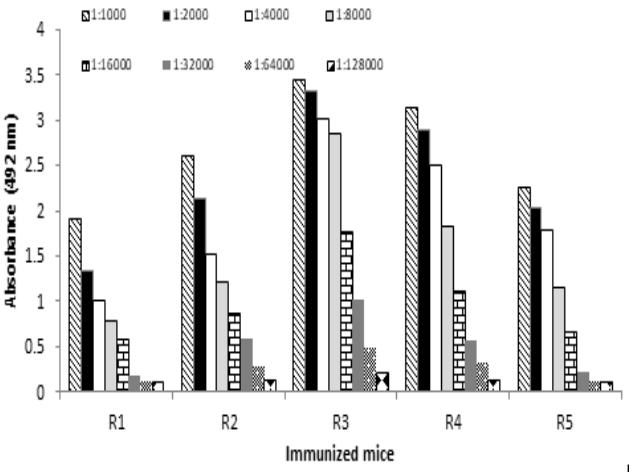

Figure 1. IgG antibody response in five mice immunized with the P22 antimicrobial peptide. Data show the absorbance values at 492 nm obtained, by means of an indirect ELISA, with serially diluted sera, from 1:1000 to 1:128000.

Five of the hybridomas with favorable cell growth, were detected as secretors of specific antibodies against P22 to the culture supernatant, with absorbance signals greater than 1.0. Not all immunization schedules, nor the immunogens used, induce the obtaining of the expected antibody [11]. In this study, it was demonstrated that, using a scheme with three subcutaneous immunizations with 28-day intervals with a final intravenously booster, together with the concentration and purity of the antigen, it was possible to obtain an acceptable fusion efficiency and the generation of hybridomas secreting specific antibodies against the antigen of interest.

The anti-P22 secreting hybridomas were expanded to 24-well plates and they maintained the ability to secrete antibodies against P22, which was verified by a second evaluation by ELISA. Three of them were selected: 2G8, 2G9 and 5A8, according to their growth characteristics in culture and for having higher absorbance signals in culture supernatants. These hybridomas were cloned by limiting dilution, during which wells containing a single colony of cells were selected. The limiting dilution method allows each well to contain at least one colony of the hybridoma of interest, thus ensuring monoclonality [8]. In addition, it has been described that some hybridomas lose the ability to secrete antibodies or their basic functions for cell viability, and they can be more metabolically efficient and dominate the culture [12]. To avoid multiplication of these non-secreting and undesired antibody hybrids, limiting dilution cloning is recommended immediately after evaluating the supernatants of the specific hybridomas.

Culture supernatants of the three selected hybridomas were evaluated by ELISA to verify the specific recognition of P22 unconjugated to BSA, as well as to detect if exist cross-reactivity of the antibodies against P21 as a related peptide. When the ELISA plate was coated with P22-BSA and with P22 unconjugated to BSA, there were no differences in the absorbance values obtained for each of the hybridomas (Fig. 2), which reveals that the signal is not due to the presence of antibodies against BSA, but against P22. The absorbance was closed to zero when the plate was coated with peptide P21 (Fig. 2), indicating that the antigen-antibody reaction did not occur in that case. This result shows that the monoclonal antibodies obtained do not show cross-reactivity with the related P21 peptide.

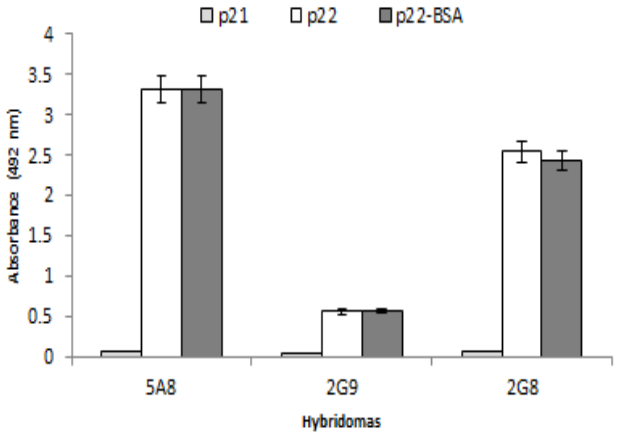

Figure 2. Evaluation of the reactivity of three anti-P22 monoclonal antibodies against P21 (related peptide) and against P22 conjugated and unconjugated to bovine albumin (BSA). The results represent the means of the absorbances obtained from three independent ELISAs.

The culture supernatant of each hybridoma was used for the determination of the class and the subclass of the antibodies that they secreted. All of them were included into IgG class (Table 1). For the complete characterization of these mAbs, other parameters such as the affinity constant and the specificity of epitopes must be analyzed in future studies.

Table 1. Class and subclass of the anti-P22 antibodies produced by the secreting hybridomas.

Monoclonal antibodies	Class/Subclass
2G8	IgG/IgG2b
2G9	IgG/IgG2a
5A8	IgG/IgG2a

The 5A8 hybridoma was selected for the production of the mAbs in ascites fluid and subsequent purification, since it had the highest absorbance signal (Fig. 2). The purification of this anti-P22 mAb was carried out by protein A affinity chromatography. An eluate was obtained with 27 mg of total IgG (3.2 mg of IgG/mL Protein A matrix), with purity higher than 95%. One of the most frequent contaminants in antibody purification is albumin, due to its high concentration in ascites. The purification process was improved with ammonium sulfate precipitation, method based on the solubility of the immunoglobulin. The mouse

IgG precipitates at 50% ammonium sulfate, while albumins remain in soluble fraction. Regarding the chromatogram of the process (Fig. 3), it was observed that the elution peak corresponded to a high purity fraction of the monoclonal antibody. The absorbance corresponding to the unabsorbed fraction indicated that clarification of ascites with glass wool and precipitation with ammonium sulfate contributed to the purity of the eluted antibody.

The SDS-PAGE profile obtained under reducing conditions showed the expected bands of 50-55 kDa and 25 kDa, representative profile of IgG heavy and light chains (Fig.4).

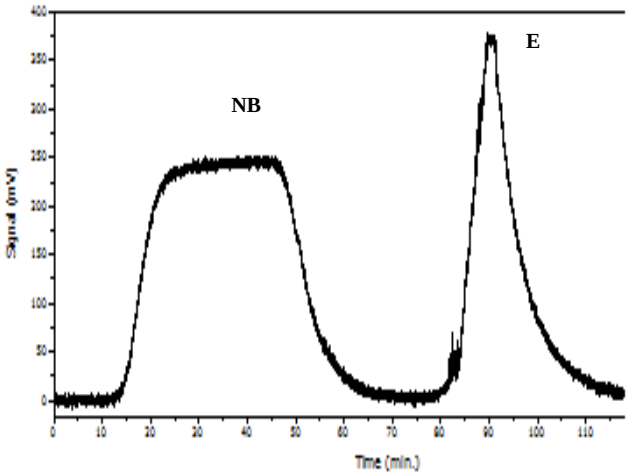

Figure 3. Chromatogram of Protein A affinity purification of anti-p22 monoclonal antibody. NB: proteins that not stick to the matrix, E: monoclonal antibody elution at pH 6.0

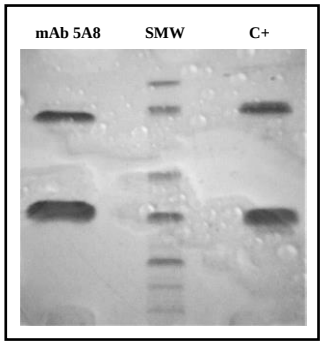

Figure 4. Polyacrylamide denaturing gel electrophoresis of Protein A affinity purification of anti-P22 monoclonal antibody. SMW: Standard Molecular Weight pre-stained SDS PAGE Standard Broad range (Biorad), C+: anti-IL2h monoclonal antibody used as positive control.

IV. CONCLUSION AND FUTURE SCOPE

Hybridomas producing mAbs were generated for the first time against the antimicrobial peptide Oreoch-2 from tilapia. These mAbs will be useful to study the mechanisms of action of this peptide, and to establish analytical tools for detection and quantification of the peptide in new pharmaceutical formulations to use it as new generation antimicrobials.

REFERENCES

[1] K. Bermúdez, G. Hidalgo, R. Mora, C. Rodríguez, B. Ysmael-Acle, J. Mora, "Anticuerpos monoclonales biespecíficos: desarrollo, producción y anticancerígena," *Revista Médica de la Universidad de Costa Rica*, Vol.**13**, No.**1**, pp.**11-29**, **2019**.

[2] R. Seyfi, F. Kahaki, T. Ebrahimi, S. Montazersaheb, S. Eyvazi, V. Tarhriz, "Antimicrobial peptides (AMPs): roles, functions and mechanism of action," *International Journal of Peptide Research and Therapeutics*, Vol.**26**, pp.**1451-1463**, **2020**.

[3] M. González, J. San Juan, F. Morales, A. Otero, "Péptidos antimicrobianos: potencialidades terapéuticas," *Revista Cubana de Medicina Tropical*, Vol.**69**, No.**2**, pp.**1-13**, **2017**.

[4] J. Acosta, V. Montero, Y. Carpio, J. Velázquez, H. Garay, O. Reyes, A. Cabrales, Y. Masforrol, A. Morales, M. Estrada, "Cloning and functional characterization of three novel antimicrobial peptides from tilapia (*Oreochromis niloticus*)," *Aquaculture*, Vol.**372**, pp.**9–18**, **2013**.

[5] J. Acosta, Y. Carpio, I. Valdés, J. Velázquez, Y. Zamora, R. Morales, A. Morales, E. Rodríguez, M. Estrada, "Co-administration of tilapia alpha-helical antimicrobial peptides with subunit antigen boosts immunogenicity in mice and tilapia (*Oreochromis niloticus*)," *Vaccine*, Vol.**32**, pp.**223– 229**, **2014**.

[6] J. Acosta, M. Estrada, Y. Carpio, "Péptidos antimicrobianos aislados de tilapia (*Oreochromis niloticus*) como agentes terapéuticos y adyuvantes moleculares para peces y mamíferos," *Revista Anales de la Academia de Ciencias de Cuba*, Vol.**4**, No.**2**, pp.**1-20**, **2014**.

[7] G. Kohler, C. Milstein, "Continuous cultures of fused cells secreting antibody of predefined specificity," *Nature*, Vol.**256**, pp.**495-497**, **1975**.

[8] J. Gavilondo, "Anticuerpos monoclonales," Elfos Scientiae, La Habana, pp.**51-55**, **1995**.

[9] Y. Machado, "Caracterización de anticuerpos monoclonales funcionales específicos de ADAM-17," Memory to qualify for the Degree of Doctor, Complutense University of Madrid, pp.**85**, **2017**.

[10] N. Amin, N. Reyes, F. Camacho, O. Otero, M. Cuello, D. Núñez, S. Fernández, L. García, "Obtención de un anticuerpo monoclonal murino que reconoce al polisacárido capsular Vi de *Salmonella Typhi*," *VacciMonitor*, Vol.**24**, No.**2**, pp.**57-63**, **2015**.

[11] G. León, C. Cruz, L. Rojas, "Producción de anticuerpo monoclonal (AcMo) con especificidad anti B, para ser utilizado como reactivo de hemoclasificación," *Investigación clínica*, Vol.**45**, No.**2**, pp.**145-157**, **2004**.

[12] J. Trejos, J. Castaño, "Obtención de anticuerpos monoclonales de ratón contra proteasa de cisteína 5 recombinante de *Entamoeba histolytica*," *Revista MVZ Córdoba*, Vol.**17**, No.**2**, pp.**3014-3023**, **2012**.

