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PRELIMINARY REPORT

Generation of Mouse Hybridomas Secreting Anti-*Salmonella* Enteritidis Antibodies and Their Preliminary Characterization [1]

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Summary

BALB/c mice were intraperitoneally immunized with inactivated bacteria for generation of monoclonal anti-*S.* Enteritidis antibody. The spleen cells of the highest responder animal at fifth immunization were used as the fusion partner of the mouse Sp2/0 myeloma cells. A total of 6 stable hybridomas secreting IgM and IgG isotype antibodies were obtained. These hybridomas were found to be reactive with three *S.* Enteritidis antigens having relative molecular weights of 73, 59 and 42kDa in Western blot analysis. The 59kDa molecule corresponds to the flagellin protein. From this preliminary study, it can be concluded that further investigations are necessary to obtain monoclonal hybrid cells secreting monoepitopic and monoisotypic antibody by subcloning of the parental hybridomas.

Keywords: *Salmonella Enteritidis, Cell fusion, Hybridoma, Specific antibody*

Anti-*Salmonella* Enteritidis Antikorları Salgılayan Fare Hibridomalarının Geliştirilmesi ve Ön Karakterizasyonları

Özet

Salmonella Enteritidis'e karşı monoklonal antikor üretmek amacıyla BALB/c fareler inaktif bakteri ile periton içi bağışıklandı. Beşinci bağışıklamada en yüksek yanıtı veren hayvana ait dalak hücreleri fare Sp2/0 miyeloma hücrelerinin füzyon partneri olarak kullanıldı. IgM ve IgG izotipi antikorlar salgılayan toplam 6 adet kararlı hibridoma hücresi elde edildi. Bu hibridomaların Western blot analizinde *S.* Enteritidis'e ait 73, 59 ve 42 kDa relatif molekül ağırlıklarına sahip 3 antijen ile reaksiyon verdiği belirlendi. 59 kDa molekül Salmonella flagellin proteinine denk düşmektedir. Bu ön çalışmada, monoepitopik ve monoizotipik antikor salgılayan monoklonal hibrid hücrelerin elde edilmesi için ilave araştırmaların yapılmasının gerekli olduğu sonucuna varılmıştır.

Anahtar sözcükler: *Salmonella Enteritidis, Hücre füzyonu, Hibridoma, Spesifik antikor*

INTRODUCTION

Salmonella enterica subsp. Enteritidis is an important pathogen causing significant economical losses in poultry and human food-borne diseases. Identification of *Salmonella ssp*. is based on bacteriological, biochemical and serological tests. The enzyme-linked immunosorbent assay (ELISA) have been preferred lately for the diagnosis of salmonellosis [1,2]. Indirect ELISAs based on the LPS and flagellin antigens were found suitable for large scale testing of flocks with more reliability [1-4]. Moreover, capture ELISAs developed by polyclonal or monoclonal antibodies have also been used to demonstrate the presence of Salmonella antigens [5]. Monoclonal antibodies (MAbs) constitute the preferred reagents in immunoassays to avoid cross-reactions by virtue of their monoepitopic specificity. The additional advantage of the MAbs is their unlimited *in vitro* standard production at desirable quantities. For reliable

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and accurate serodiagnosis of avian Salmonellosis, several studies have been undertaken to generate monoclonal antibodies directed against immunodominant O somatic and H flagellin antigens of different Salmonella serotypes [5-9]. In this perspective, the aim of the current study was to generate mouse hybridomas producing anti-*S.* Enteritidis antibodies and to characterize their isotypes and antigenic counterparts.

MATERIAL and METHODS

A *Salmonella enterica* subsp. Enteritidis strain (SE/OMU.05.005) from culture collection of Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Microbiology was used. Its antigenic formula was determined in an earlier study [4] as O1, 9, 12 and gm:- by using mono-specific O4 and O9 as well as Hg and Hm antisera (Denka Seiken, Japan). *S.* Enteritidis was grown overnight in Brain Heart Infusion Broth and inactivated by the addition of formaldehyde (0.5%). Bacterial lysate was prepared by heating (100ºC for 15 min) the bacteria resuspended in lysis buffer [125 mM Tris-Cl, 150 mM NaCl, 2% sodium dodecyl sulfate (SDS), pH 6.8]. After centrifugation (25.000 g, 30 min), the dialyzed supernatant was used as total bacterial antigen (LYS).

For hybridoma generation, 3 female BALB/c mice (6 to 8 weeks old) were intraperitoneally immunized with 10^8 formaldehyde-inactivated *S.* Enteritidis cells prepared in a volume of 0.5 ml of PBS emulsified with an equal volume of Complete Freund Adjuvant (CFA, Sigma - Aldrich). At two week intervals, intraperitoneal immunizations were repeated four times using 10^8 inactivated bacteria emulsified in PBS-Incomplete Freund Adjuvant (IFA, Sigma - Aldrich). Blood samples were taken at day 0 and 14 days after each immunization. The animal with the highest titer of specific antibody was boosted with 10^8 inactivated *S.* Enteritidis cells. After three days, mouse spleen cells were harvested and fused with Sp2/0 mouse myeloma cells by the addition of PEG (polyethylene glycol) at a ratio of 5:1 in DMEM (Dulbecco's Modified Eagles Media containing 200 mM L-glutamin and 100 U/ml of penicilline-streptomycin mixture) according to the method of Harlow and Lane [10]. After centrifugation (400 x g, 10 min), the cells were resuspended in HAT (hypoxantine-aminopterin-thymidine) supplemented DMEM containing 20% FCS (Gibco). They were dispensed into the wells of 96-well culture plates preincubated with a feeder layer of $5x10^4$ mouse spleen cells per well and cultured for 10-14 days under 5% CO_2 atmosphere. Then, the medium was replaced by HT (hypoxantine-thymidine) supplemented DMEM. Hybridomas producing anti-Salmonella antibody were determined by ELISA using their culture supernatant. They were frozen and stored in liquid nitrogen.

ELISA was performed as previously described [4]. Briefly, 96-well microtiter plates were coated with inactivated bacteria (10^7 cfu/well) in 0.05 M carbonate buffer. Diluted mouse sera or undiluted hybridoma culture supernatants (100 µl) were added to the wells and incubated at 37°C for 1 h. Anti-mouse Alkaline Phosphatase conjugated antibodies (Sigma-Aldrich) were added and incubated for 1 h at 37°C. The development of the reaction was evaluated by the addition of 100 µl of pNPP substrate (Sigma - Aldrich Inc.). The optical density (OD) was measured at 405 nm in ELISA reader (Thermo Multiskan). The samples were tested in duplicate.

Isotype and antigenic counterpart determinations of the reactive hybridomas were performed by Western blot analysis. For this purposes, whole cell extracts (LYS) of *S.* Enteritidis were separated on 10% polyacrylamide gels according to the method described by Laemmli [11] and stained with coomassie brilliant blue or electro-transferred to polyvinyldifluoride (PVDF) membranes (Immobilon-P, Sigma - Aldrich) according to the method described by Towbin et al.[12]. After blocking with 1% skimmed milk in PBST, the membrane strips were incubated with either pre-immunized or immunized mouse sera diluted 1/1.000 and 1/5.000 respectively and undiluted hybridoma culture supernatants for 90 min at room temperature. The membranes were then incubated with AP conjugated goat anti-mouse gamma (γ) or mu (µ) chain specific antibodies (Sigma - Aldrich) for 1 h at room temperature in order to determine antibody isotypes. Color reaction was developed with the addition of BCIP/NBT-blue liquid substrate system for membranes (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, Sigma - Aldrich) and stopped by washing with distilled water.

RESULTS

Three female BALB/c mice immunized with *S.* Enteritidis cells have developed detectable humoral immune response in ELISA following the second immunization. The antibody response became important after the third immunization and gradually increased by fourth and fifth immunizations. One of the immunized mice reached the highest antibody response (1/25.000 ELISA titre) after the fifth immunization. The spleen cells of that animal were used as fusion partners for the generation of monoclonal anti-*S.* Enteritidis antibody. Two weeks after the fusion, 36 parental hybridomas were obtained. The supernatant of 9 hybridoma cell cultures gave positive reaction in ELISA. Among these hybridomas, only six (designated as 3D1, 2H3, 1G8, 2A11, 1H11, and 1H3) were found growing and producing anti-Salmonella antibody during one month of culture. To determine antigenic molecules of *S.* Enteritidis recognized by these hybridomas, Western blot analysis was performed by using alkaline phosphatase conjugated antisera to IgM and IgG isotypes (µ- and γ-chains specific, respectively) as detector reagents.

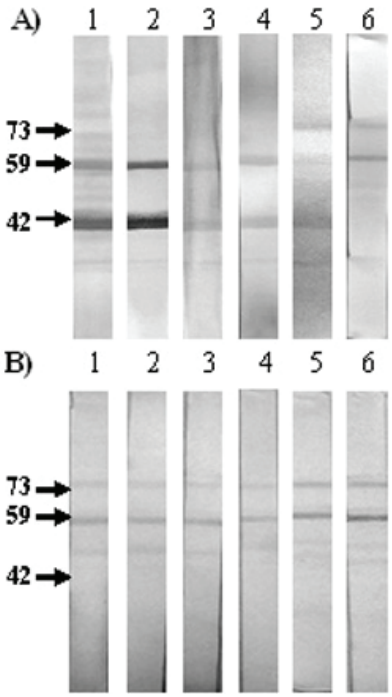

Fig 1. Western blot analysis of the hybridomas using *Salmonella* Enteritidis lysate as diagnostic antigen

Strips 1-6: The hybridomas designated as 3D1, 2H3, 1G8, 2A11, 1H11 and 1H3, respectively. Panel A shows IgM and Panel B indicates IgG isotype antibodies. The arrows show the recognized major *Salmonella* Enteritidis antigens

Şekil 1. Tanı antijeni olarak *Salmonella* Enteritidis lizatının kullanıldığı hibridomalara ait Western blot analizi

Strip 1-6: Sırasıyla 3D1, 2H3, 1G8, 2A11, 1H11 ve 1H3 olarak adlandırılan hibridomalar. Panel A IgM ve Panel B IgG izotipi antikorları göstermektedir. Oklar tanınan major *Salmonella* Enteritidis antijenlerini göstermektedir

The results presented in *Fig. 1A* demonstrated that all parental hybridomas secreting IgM isotype antibodies recognized at least two protein bands having relative molecular weights of 73, 59 and 42kDa. Four parental hybridoma supernatants (3D1, 2H3, 1G8 and 2A11) reacted with protein bands of 59 and 42kDa while 1H11 hybridoma recognized a third additional band of 73kDa *(Fig. 1A)*. 1H3 hybridoma has been found to secret IgM isotype antibodies reacting with 73 and 59kDa molecules. When the presence of IgG isotype antibody was investigated by Western blot analysis, two major protein bands of 73 and 59kDa were found to be recognized *(Fig. 1B)*. Although all of the hybridomas were detected to be more reactive with 59kDa protein, higher reactivity was observed with 1H3 and 1H11 hybridomas.

DISCUSSION

The accurate and rapid detection of *Salmonella* Enteritidis from poultry products is important for public health and development of rapid and specific diagnostic assays based on the monoclonal antibody will be useful for this purpose. In this perspective, the present study aimed to generate hybridoma cells producing anti-*S*. Enteritidis antibodies. In this study, we have obtained a total of 36 parental hybridomas and 9 of them (25%) were found to be antibody secreting cells. These results indicated that a high ratio of specific antibody secreting hybridomas can be obtained by using as fusion partner the spleen cells of the mouse with the highest antibody titer following multiple immunizations with inactivated bacteria. Being obtained only IgM and IgG isotype anti-*S*. Enteritidis antibodies in the present study can be explained by the fact that intraperitoneal immunization by inactivated bacteria essentially stimulated the generation of IgM and IgG isotype antibodies. This interpretation are strongly supported by the study of Luk and Lindberg [6] where several monoclonal anti-*Salmonella* LPS antibodies obtained by intraperitoneal immunization with killed bacteria were of IgM and IgG isotypes only. Nevertheless, Iankov et al.[9] obtained a significant yield of hybrid cells secreting IgA (16%) and a total of 30 hybridomas producing IgG and IgM isotype antibodies by using oral immunization approach with live *Salmonella* Suberu. These results showed that immunization route and physiological status of the bacteria used as immunogen are critical parameters for ensuring the generation of hybridomas secreting antibodies belonging to different isotypes.

In our study, 6 hybridomas were found to be secreting simultaneously IgM and IgG isotype antibodies after one month of the culture. This indicated the presence of at least two clonal cells in 6 parental hybridomas. In fact, a parental hybridoma (1H11) recognized three bands of relative molecular weight of 73, 59 and 42kDa in IgM-based Western blot analysis *(Fig. 1A)* while 4 others reacted with 59 and 42kDa molecules and 1H3 hybridoma was found reactive with 73 and 59kDa bands. In the IgG-based Western blot analysis *(Fig. 1B)*, two major protein bands of 73 and 59kDa were recognized by all of the 6 hybridomas. Among these three reactive bands, the 59kDa molecule corresponds to the flagellin, subunit of bacterial flagella, as demonstrated in earlier studies [4,13,14]. On the contrary, 73 and 42kDa molecules recognized by various hybridomas were not yet identified.

This study allowed obtaining 6 stable parental hybridomas secreting IgM and IgG isotype antibodies more or less reactive with three *S*. Enteritidis molecules. These results indicate that the parental hybridomas contain more than one clone. From this preliminary study, it can be concluded that further investigations are necessary to obtain monoclonal hybrid cells secreting monoepitopic and monoisotypic antibody by subcloning of the parental hybridomas.

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