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Production of Monoclonal Antibodies against Common Epitopes of Salmonella by Oral Immunization of Laboratory Animals

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Abstract

Materials and Methods: Twelve mice were divided into two groups and immunized orally with *S. suberu* (3.10: g, m) and *S. strasburg* from the OD2 group (O:9.46). The Köhler and Milstein method yielded IgA and IgM class IgA secreting monoclonal antibodies. They were characterized by the methods of slide-agglutination, haemagglutination, ELISA, immunoblotting.

Results: Four anti-flagelar MAbs of class IgA and one anti-O-class IgM class were obtained. All of the IgA class reacted with the Hg epitope of flags obtained from *Salmonella* strains and *S. enteritidis* flagellin. In IgMMAbs, only O-antigens isolated from *Salmonella* strains belonging to the OD2 group were present.

Conclusion: Through this method of oral immunization, besides highly specific IgA MAbs, we also received a clone of hybridomas expressing specific IgM MAbs which by ELISA and SAT show excellent opportunity to participate in diagnostic reactions against salmonellae with a common group epitope OD2.

Keywords: Monoclonal antibodies; *Salmonella*; Oral immunization

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Introduction

Salmonella are Gram-negative bacteria of the Enterobacteriaceae family. Two species have been described – *Salmonella enteric* and *Salmonella bongori*. *S. enterica* is a type, which is divided into six subspecies, consisting of over 2500 serotypes. For convenience in clinical practice, serotypes are often named as species. *Salmonella* does not form spores; they are also heterotrophs used for nutrients using organic sources. Serotypes of Salmonella enteric are spread all over the world among animals and humans, some of which are disease-causing. The most common diseases caused by *Salmonella* bacteria are *S. typhi* and a number of gastroenteritis and food toxic infections – *S. enteritidis, Salmonella group D*, etc. [1,2].

For the purposes of the serological classification of *Salmonella*, Kauffmann-White scheme was required, which subdivided them into several serogroups from A to O:67 depending on the structure of their lipopolysaccharides (O-antigens) and H-antigens, called crunchy antigens [1]. The most frequent differentiation of individual serogroups and subgroups is by polyclonal monospecific rabbit sera. Monoclonal antibodies (MAbs), however, have many advantages due to their monoepitopic specificity and therefore high affinity for the respective epitope. Multiple diagnostic monoclonal antibodies against the H- and O-antigens of some salmonellaes have been developed by intraperitoneal immunization of laboratory animals [2-6]. Similar experiments lead to the production of immunoglobulins M, G, and we have results in the production of immunoglobulins A [7-9]. Oral (intragastric) immunization of experimental animals to produce antibodies other than IgA class [9,10] is still rarely performed.

Objective

The purpose of this work is to obtain diagnostic monoclonal antibodies of different classes against common epitopes of salmonella bacteria by oral immunization of laboratory animals.

Materials and Methods

Salmonella strains and purified antigens

The *Salmonella* strains used were provided by the National Bank for Microorganisms and Cell Cultures – Bulgaria, Pasteur Institute – France, NCIPD Collection – Sofia, Bulgaria. Parts of the strains are from the Department of Microbiology at the Sofia Medical University, we also used clinical strains of *S. enteritidis* from the University Hospital for Infectious and Parasitic Diseases "Prof. Iv. Kirov" – Sofia, Bulgaria. LPS of the strains were purified by the phenol-water method of Westphal and Jann [11]. H-antigens were purified according to Ibrahim's method [7].

Production of monoclonal antibodies

Six BALB/c mice were immunized orally (intragastrally, i.g.) with 109 heat-inactivated bacteria from *Salmonella suberu* strain (3.10: g, m) and six with *Salmonella strasbourg* from group OD2 (O:9.46) in both cases, the bacteria were diluted in 0.5 mL carbonate bicarbonate buffer at pH 9.6. Immunization is repeated on the 14^{th} and 28^{th} day. After 2 weeks, blood was taken of mice and the serum was tested by slide-agglutination. Two animals of each group with an apparent highest titre of specific antibodies were boosted intravenously with 109 formalin-killed bacteria. After 72 h, the spleen cells were harvested and fused with X63-Ag8.653 myeloma cells according to the Köhler and Milstein method [12]. Hybridomas were cultured in 24-well plates (Costar) in Eagle's medium modified by Dulbecco (Sigma), supplemented with 1-glutamine hypoxanthine-aminopterin-thymidine and 10% fetal bovine serum (Gibco). On day 14, supernatants were screened by antigen-mediated ELISA. Positive wells were cloned by limiting dilution and isotyped by antigen-mediated ELISA using an ISO-2 kit (Sigma).

Characterization of antigenic specificity of monoclonal antibodies

ELISA: Plaques were loaded with flagellar antigens (1 µg per well diluted in 100 µL acetate buffer pH 5.6). After overnight incubation at room temperature, the plates were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20. After blocking with 0.5% bovine serum albumin (BSA) in PBS at 37°C for 2 hours, diluted sera or undiluted hybridoma culture supernatants were added to the wells. The plates were incubated at 37°C for 1 hour, washed and the secondary anti-mouse polyvalent immunoglobulin (G, A, M) peroxidase-conjugated antibody (Sigma) was added for 1 hour. The enzyme reaction was developed with 0.04% H_2O_2 and 0.04% O-phenylene diamine in pH 5.0 phosphate-citrate buffer and terminated after 20 minutes by the addition of 25 µL of 4N H_2SO_4 per well. Optical density was measured at 492 nm on a MRX microplate reader (Dynatech Laboratories) and values above 0.100 were considered positive.

SDS-PAGE immunoblot

Purified *Salmonella* flagellines are denatured in assay buffer under reducing conditions and separated by SDS-PAGE in a 10% gel [13]. The immunoblot was performed according to the Towbin method [14]. Fractionated *Salmonella* flagellines are transferred from the separating gel to a polyvinylidene fluoride membrane (Millipore) for 1 hour at 0.8 mA cm^{-2} of the gel area using a Bio-Radsemi-dry blotting system. After blocking with 0.5% BSA in PBS/T, the membranes were incubated overnight at 4°C with hybridoma culture supernatants. Immunoblot is visualized using an anti-mouse IgA (a-chain specific) peroxidase conjugate (Sigma) and 4-chloro-1-naphthol (Sigma) as peroxidase.

To demonstrate the immunomodal and polymeric IgA forms, the purified MAbs of each hybridoma clone were separated by SDS-PAGE under reducing and non-reducing conditions and analyzed by immunoblotting with a secondary antibody peroxidase-conjugated a-chain murine-specific antibody [9,10].

Slide agglutination (SAT)

Supernatants of hybridoma culture were tested in SAT with different strains of *Salmonella* and a large number of clinical isolates. The assay was performed by mixing 25 µL of antibody dilutions with medium grown bacteria and the results were recorded for 2 min. Prior to SAT, the *Salmonella* strains of antigens were tested with commercial anti-H salmonella sera, and for the O-antigens a haemagglutination reaction was used in which sheep erythrocytes were loaded with purified LPS-antigens (Difco Laboratories, USA and BulBio NCIPD, Ltd. Bulgaria) [8,9].

Results

Six female BALB/c mice were immunized intragastrally (orally) with heat-inactivated *S. suberu* (3.10: g, m) and the other six with *Salmonella strasbourg* (O:9.46). From each group, two of the animals with the highest level of specific antibodies in the serum (1:320 titers in SAT) were also immunized intravenously once. After screening the produced hybridomas, four anti-flagelar MAbs of the IgA class and one anti-O-class IgM class were obtained.

MAbs were characterized by ELISA with a large number of purified *Salmonella* flagellines. All of the IgA class reacted with the Hg epitope of flagellines obtained from *Salmonella* strains with H:g, m, H, g, m, s, H, g, H, g: and H: f, g, s specificities. They all responded to *S. enteritidis* flagellin. The MAbs we have received are agglutinating in SAT all 62 clinical isolates of *S. enteritidis* available in our laboratory collection.

Characterization of the resulting IgM MAbs was performed by a number of purified LPS antigens by SAT and ELISA **(Table 1)**, and it only reacted with the O-antigens isolated from salmonella strains belonging to the OD2 group. This method is able to detect 10 ng mL^{-1} purified antigen and 105 mL^{-1} bacteria in the samples [2,8,9,13].

Discussion

The production of IgA secreting hybridomas is relatively difficult because it requires mucosal administration of the antigen. IgA is the major isotype of the lining that is produced by local plasma cells [9,10]. Oral immunization with live microorganisms and subsequent fusion of myeloma cells and isolated lymphoblasts from the Peyer patch and is the main way to produce monoclonal

Table 1 ELISA for the monoclonal antibody after *Salmonella strasbourg* immunization.

Monoclonal antibody	Purified LPS group OD2
Group 1	
102D12	-
198F10	-
Group 2	
192A4	1.801
1.93E+11	0.724
194g1	1.023
195D11	1.281
196H1	1.287

IgA antibodies [9,10,13]. The other strategy is the selection of IgA isotype switching variants of IgM or IgG secreting hybridomas [10]. The fusion between spleen lymphoblasts and myeloma lines after mucosal administration of antigen is also effective in the production of IgA MAbs. In our work, immunization with live (inactivated) *S. suber* and *S. strasbourg* and further i.v. enhancing the immunization with killed bacteria followed by spleen fusion has been shown to generate not only specific IgA secreting hybridoma clones but also IgM secreting. The strains of *S. enteritidis*, as the most common antigen in humans, are virulent for mice and cause generalized infection after i. g. application of live bacteria, so the method with them is not applicable. Therefore, we have chosen to immunize *S. suberu* and *S. Strasbourg*, which are not virulent for mice.

Conclusion

We believe that by this oral immunization method, in addition to highly specific IgA MAbs, we have also received a clone of hybridomas expressing specific IgM MAbs which by ELISA and SAT also show an excellent opportunity to participate in diagnostic reactions against salmonellaes with a common group epitope OD2.

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