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Production of Monoclonal Antibodies to Vi Polysaccharide Antigen of *Salmonella typhi*

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SUMMARY **Twenty-four Vi antigen-specific monoclonal antibodies were produced in this study. The MAbs were found to be highly specific to Vi possessing bacteria. Selected MAbs were used in a direct agglutination assay for rapid identification of *S.typhi* in primary bacterial culture and also used to develop an assay to detect Vi antigen in clinical specimens. The result showed that they could not detect the antigen in urine and serum from acute patients even they could detect as low as 0.02 μg/ml of Vi antigen added in normal urine. The study has shown that these MAbs are very useful for rapid identification of *S.typhi* in primary bacterial culture and they can replace polyclonal anti-Vi antibodies which have been used routinely in bacteriological laboratories.**

Typhoid fever, a disease caused by *S.typhi* remains an important infectious disease problem in many countries around the world.[1] The identification of the causative agent is useful for the epidemiological investigation of the source of infection.

Classical identification of *S. typhi* involves culture on primary agar plates and requires another 24 hours for biochemical testing. The colonies suspected to be Salmonellae will be further tested with their specific antisera. Most isolates of Salmonella from natural sources can be classified serologically into five groups, A to E, by a combination of multiple O (somatic), H (flagella) and Vi (virulence) antigens. *S.typhi* is allocated to group D.[2] The Vi (virulence) antigen is a capsular polysaccharide antigen which is present in the outermost layer of *S.typhi*.[3] This antigen is presented in limited strains of bacteria, such as *S.typhi, S.paratyphi* C, *C.freundii* and some strain of *S.dublins*. Among these 4 bacteria, only *S.typhi* and *S.paratyphi* C can cause enteric fever. A polyclonal monospecific serum against Vi antigen is routinely used to differentiate *S.typhi* from other Salmonellae in serogroup D. Production of polyclonal monospecific antisera against Vi antigen requires extensive absorption and lot-to-lot variation in specificity and efficacy makes it difficult to standardize. Monoclonal antibody against Vi antigen should not have these problems and can potentially replace polyclonal monospecific antisera.

In the present study, MAbs against Vi capsular polysaccharide antigen were established. These MAbs were able to differentiate *S. typhi* from other Salmonellae as tested by slide agglutination. This technique can be used to identify *S.typhi* from stool culture plate by direct agglutination between MAb and a single bacterial colony which can be used in rapid identification of *S.typhi* in screening of chronic carriers of typhoid fever.

MATERIALS AND METHODS

Production of monoclonal antibody

Five micrograms of purified Vi capsular polysaccharide of *S.typhi* (provided by Dr Patrick Poirot,

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Institut Merieux, Lyon, France) in 100 μl normal saline solution were injected intraperitoneally into BALB/cJ mice. Booster injections with the same dose and route were given after 10 days. Three days later the spleen was removed and fused with a myeloma cell line (P3X63 Ag8.653). The fusion procedure was carried out as described by Kearney *et al.*[4] After hybrid clones were selected, the culture supernatants were tested for antibody activity by indirect ELISA Positive hybridomas were cloned three times by using limiting dilutions to establish stable clones.

Screening for specific anti-Vi antibodies

Indirect ELISA employing purified Vi capsular polysaccharide antigen of *S.typhi* coated plates was used. Five micrograms per millilitre of Vi antigen were diluted in carbonate buffer (pH 9.8) at 37°C for 3 hours. After washing the plate with 0.05% v/v Tween 20 in normal saline solution (Tween saline) the culture supernatant was added and incubated at 4°C overnight. Alkaline phosphatase conjugated rabbit antimouse immunoglobulin (Dakopatts, Denmark) diluted in 1% bovine serum albumin in Tween-saline was then added and incubated for 3 hours at 37°C. After excess conjugate was removed, the substrate solution containing 1 mg/ml p-nitrophenyl phosphate (Sigma, USA) in substrate buffer was added. The enzymatic reaction was allowed to proceed at room temperature for 1 hour. The optical density was measured at 405 nm with Titertek Multiskan (Flow Laboratory, Germany).

Characterization of monoclonal antibody

The isotype of monoclonal antibody was characterized by indirect ELISA. Alkaline phosphatase conjugated goat antimouse IgM, IgG, IgG2a, IgG2b, IgG3 (Southern Biotechnology, USA) were used.

Direct agglutination test for identification of *S.typhi* colony

Twenty microliters of ammonium sulfate precipitate of selected monoclonal antibodies at 5 mg/ml concentration were mixed with one colony of bacteria from a culture plate on a glass slide and rotated. The agglutination was recorded by eye within 2 minutes.

Antibody sensitized protein-A bearing *Staphylococcus aureus*

One hundred microliters of ammonium sulfate precipitate of monoclonal antibodies at 10 mg/ml were added to 1 ml of 10% protein-A bearing *S.aureus* (kindly provided by Dr Nils Carlin, Department of Vaccine Production, National Bacteriological Laboratory, Sweden). The mixture was kept at room temperature for 3 hours under continuous agitation. The suspension was washed in PBS twice by centrifugation. The bacteria was resuspended to 2% (v/v) in PBS and stored at 4°C until used.

Antibody sensitized latex particle

One ml of ammonium sulfate precipitate of monoclonal antibodies at 2 mg/ml was added to 1 ml of 1% 0.793 μ sulfonated latex beads (Interfacial Dynamics Corporation, Portland, Oregon USA). The mixture was mixed by gentle stirring overnight at room temperature. BSA was added to a concentration of 2.5% and mixed for an additional 4 hours. The suspension was washed 4 times by centrifugation then resuspended to 1% (v/v) in GBS (0.1 M glycine, 0.15 M NaCl pH 8.0) and 2% glycerol, and store at 4°C until used.

Slide agglutination test for detection of Vi antigen

Twenty microliters of sensitized *S. aureus* or latex particles was mixed with 20 μl of varying concentrations of Vi antigen of *S.typhi* added in normal urine, serum or urine samples from patients. The mixture was rotated for 2 minutes. The agglutination was recorded by eye within 2 minutes.

RESULTS

Specificity of monoclonal antibodies for Vi capsular polysaccharide antigen of *S.typhi*

Twenty-four hybrid clones that reacted with purified Vi antigen of *S.typhi* were generated. All monoclonal antibodies were highly specific to the Vi antigen and reacted with Vi possessing bacteria such as *S.typhi, S.paratyphi* C, some strain of *S.dublin* and *Citrobacter freundii* but gave negative results by indirect ELISA with other Salmonellae such as *S. paratyphi* A, *S.paratyphi* B, *S.choleraesuis, S.enteritidis, S.panama, S.krefeld, S.typhimurium,* and other bacteria that cause enteric fever-like illnesses such as *E.coli, Y.enterocolitica.* These MAbs were found to be of IgG1, IgG2a, IgG2b, IgG3 and IgM isotypes.

Direct agglutination for identification of *S.typhi* colony

Ten hybridoma clones were used in the experiment. The MAbs were tested against a panel of clinical isolates of various bacteria from clinical specimens (blood, stool, urine). Eight monoclonal antibodies could agglutinate 16 to 18 isolates of *S. typhi.* They did not agglutinate any of the 20 isolates of *S.paratyphi* A, 16 isolates of Salmonella group B (*S.paratyphi* B biovar Java 9 strains, and one each of *S.stanley, S.saintpaul, S.heidelberg, S.schwarzengourd, S.derby, S.agona, S.typhimurium),* 8 isolates of Salmonella group C other than *S.paratyphi* C (*S.blockley, S.potsdam, S.infantis, S.virchow, S.brunei, S.emek, S.tradar, S.montevideo),* 3 isolates of Salmonella group D other than *S.typhi (S.eastbourne, S.panama, S.enteritidis).* Eight clones which had IgM(1 clone), IgG1

(1 clone), IgG2a(5 clones), IgG2b(1 clone) isotypes provided good agglutination within 2 minutes, but the IgG3k(2 clones) isotype could not agglutinate *S.typhi.* The monoclonal antibody which provided the best agglutination reaction was IgM isotype. Moreover, it was found that these eight monoclonal antibodies could be stored at 4°C for at least 1 month.

Slide agglutination test for the detection of Vi antigen in clinical specimens

Ten hybridoma clones were used in the experiment. The efficiency of MAb to couple to protein A was investigated. It was found that IgG2a, IgG2b MAbs could be coupled to protein A very well, while IgG3b, IgM provided moderate coupling, and IgG1 could not be coupled at all. Clone Vi-G2a3 (IgG2a isotype) gave the best sensitivity (detected as little as 0.02 μg/ml purified Vi antigen suspended in normal urine). The specimens employed in this study were 55 urine samples from acute patients who had hemocultures positive for *S. typhi,* 41 urine samples from patients who had fever but hemoculture negative for any bacteria. The result showed that all of urine specimens were negative for Vi polysaccharides antigen by our slide agglutination test.

Similar results were obtained when these MAbs were used to establish a latex agglutination for detection of Vi antigen in both urine and serum samples from the same group of patients. No positive results could be demonstrated, although the test could detect readily as low as 0.02 μg/ml of Vi antigen.

DISCUSSION

MAbs obtained in the present study were comprised of all immunoglobulin classes (IgG1, IgG2a, IgG2b, IgG3, IgM), which is an advantage for developing the immunoassay. In previous work by 2 other investigators,[5,6] all Vi specific hybrid cell lines produced by one investigator were IgM class, and by the other investigator were IgG class.

The Vi monoclonal antibodies generated in the present study were shown to be highly specific to *S. typhi,* as tested in a direct agglutination test. The MAbs were able to identify 16/18 isolates of *S. typhi.* The failure of two of the 18 isolates of *S. typhi* from clinical specimens to be agglutinated by these Vi monoclonal antibodies may be related to a decreased amount or disappearance of Vi antigen after several subcultures. One of these 2 strains did not agglutinate while the other showed very weak agglutination when reacted with anti-Vi polyclonal antibody. These Mabs did not react with any other bacteria, including *S.paratyphi* A (20 isolates), *S.paratyphi* B biovar Java (9 isolates) Salmonella group B other than *S.paratyphi* B (7 isolates), Salmonella group C other than *S. paratyphi* C (8 isolates), Salmonella group D other than *S. typhi* (3 isolates) but they could react with *S.paratyphi* C and *C.freundii* which also possessed Vi capsular polysaccharide antigen.

Although *S. typhi, S.paratyphi* C and *C.freundii* are known to express Vi Ag, only patients infected with *S. typhi* and *S.paratyphi* C showed a clinical picture of typhoid fever. *S.paratyphi* C infection is very rare and the organism has not been isolated from clinical specimens for more than 20 years. In addition, *S.paratyphi* C also responds to the same antibiotic therapy as *S. typhi. C.freundii* is considered to be an opportunistic organism which has never been shown to cause an illness with a clinical picture resembling typhoid fever. Hence, it seems not to give a disadvantage with our direct agglutination test for identification of Vi positive bacterial colonies.

Conventional methods for detection of chronic carriers of *S. typhi* require identification of *S.typhi* from repeated stool cultures, which involve primary cultures of stools, biochemical identification of subculture and subsequent serological tests. For the purpose of screening chronic carriers in target groups such as food handlers, identification of the organism in the primary stool culture plate can be quickly and easily done with the Vi MAb. In the present study, rapid identification of *S. typhi* from primary culture was demonstrated with the use of anti-Vi MAbs in a slide agglutination assay.

Although the slide agglutination with MAbs employed in this study could considerably shorter the time required for identification of *S. typhi,* it still requires culture of the organisms. Therefore, attempt was made to identify Vi Ag directly from clinical specimens. The detection of Vi Ag in serum and urine using polyclonal anti-Vi antibody was reported by several investigators.[7-10] However, those results were not very specific, possibly due to the non-specificity of polyclonal Vi antibody used in the system. An attempt to detect Vi antigen in clinical specimen by using monoclonal anti-Vi antibody was performed in our laboratory. The slide agglutinations employing MAb sensitized protein-A bearing *Staphylococcus aureus* or latex particles were used to detect Vi antigen in both urine and serum samples. A high sensitivity was obtained when tested with purified Vi antigen added in normal urine. Unfortunately it failed to detect Vi antigen in both urine and serum specimens of the patients, even if urine specimens were concentrated 20 times. The failure to detect Vi antigen in urine and serum of the patients by these MAbs may be due to the very tiny amount of the antigen present or perhaps the epitope of Vi antigen was changed after being metabolized and could not be recognized by these MAbs.

In conclusion, the established Vi monoclonal antibodies are highly specific to Vi possessing bacteria, and can replace anti-Vi polyclonal monospecific antisera raised in animals for rapid and easy identification of *S. typhi* in cultured specimens.

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