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# Production of Vi Monoclonal Antibodies and Their Application as Diagnostic Reagents

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**Serum antibodies to Vi antigen were detected in mice immunized with the purified antigen but not with Vi-bearing *Salmonella typhi* whole cells. Fusion of the spleen cells from one of the Vi antibody-producing mice with NSI myeloma cells produced four stable hybridomas that secreted antibodies to Vi. Monoclonal antibodies from these four clones were all of the immunoglobulin G class and, as determined by competition, appeared to have the same epitope specificity. Despite their immunoglobulin G nature, mouse ascitic fluids induced by one of the hybridomas strongly agglutinated the Vi-positive strains of *S. typhi*, *S. dublin*, and *Citrobacter* strain 5396/38. Thus, 10 clinical isolates of *S. typhi* but not 98 strains of other bacteria were reactive in slide agglutination tests with the monoclonal antibodies.**

Vi antigen is present in almost all clinical isolates of *Salmonella typhi* (6, 8), and its detection by slide agglutination is routinely done for the identification of *S. typhi*. Demonstration of its presence in the urine or serum of patients by various immunological methods has also been described as a diagnostic test for typhoid fever (3, 23, 24). We have therefore tried to raise monoclonal antibodies (MAbs) to Vi antigen by immunizing BALB/c mice with whole cells of *S. typhi* and purified Vi antigen. In this communication, we report the serological response of mice to Vi antigen upon immunization, the production and characterization of MAbs to Vi antigen, and their application in the identification of *S. typhi*.

## MATERIALS AND METHODS

**Bacterial cells and Vi antigen.** The bacterial strains used in this study and their characteristics are given in Table 1. Clinical isolates of *Salmonella* spp. and other gram-negative bacteria not listed in Table 1 were obtained from our clinical microbiology laboratory and were identified by standard biochemical and serological methods (21). All bacteria were grown in static cultures in brain heart infusion broth (Oxoid Ltd., London, England) for 16 to 18 h at 37°C, except for those used in the slide agglutination test, which were grown on blood or nutrient agar plates. Cells from broth cultures were inactivated with 0.5% Formalin before centrifugation at 5,000 × *g* for 15 min. Bacterial cells thus harvested were washed twice with sterile phosphate-buffered saline (PBS).

The highly purified Vi antigen used in this study was a gift from J. B. Robbins, National Institutes of Health, Bethesda, Md., and was prepared from *Citrobacter* strain 5396/38 by the method of Wong and Feeley (26).

**Immunization of mice.** BALB/c mice were immunized with either *S. typhi* 620Ty whole cells or purified Vi antigen. The dosage and schedule of immunization are given in Table 2. Mice were bled via the intraorbital vein before and after immunization, and antibodies to Vi antigen in their sera were titrated against purified Vi antigen by passive hemagglutination (PHA) and enzyme-linked immunosorbent assay (ELISA).

**Production and characterization of MAbs.** MAbs were produced by the method of Köhler and Milstein (17), and fusion of cells was done by using polyethylene glycol (molecular weight, 4,000; BDH, Poole, England). Hybridomas producing Vi antibodies were screened by ELISA with purified Vi antigen; those producing specific antibodies were cloned twice by limiting dilution. Purified hybridomas were expanded for antibody production either in 250-ml tissue culture flasks (Falcon; Becton Dickinson Labware, Oxnard, Calif.) or by intraperitoneal injection into mineral oil-primed mice. MAbs from culture supernatants or mouse ascitic fluids were partially purified by precipitation with 50% ammonium sulfate. The immunoglobulin classes and subclasses and the light-chain patterns of the MAbs were determined by double immunodiffusion with anti-mouse immunoglobulin antisera (Nordic Immunological Laboratories, Tillburg, The Netherlands).

**PHA.** PHA was carried out by previously described procedures (4). Results were read both macroscopically and microscopically.

**ELISA and competitive ELISA.** Antibodies were measured by ELISA (7) with purified Vi antigen coated onto ELISA microtiter plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, Va.) at a concentration of 10 μg/ml diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Antigen adsorption was performed at 4°C overnight. Sera from mice or MAbs produced by hybridomas were diluted with 5% bovine serum albumin (BSA) in PBS. The binding of antibodies to Vi antigen was detected with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (heavy- and light-chain specific; Tago Inc., Burlington, Calif.) and *o*-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.) as the substrate. Incubation was done at 37°C for 2 h. Other relevant steps in the ELISA procedure included blocking of excess binding sites on the microtiter wells after antigen coating with 5% BSA in PBS for 1 h at 37°C and four washing with 0.85% saline–0.05% Tween 20 (saline-Tween) after antigen coating and serum incubation. Wells coated with BSA instead of Vi antigen and wells coated with Vi antigen and tested with normal mouse serum instead of immune mouse serum or MAb were included in each plate as controls.

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TABLE 1. Bacterial strains used for immunization and specificity testing

| Species | Source | Presence (+) or absence (−) of Vi antigen | Other properties |
|---|---|---|---|
| *S. typhi* 620Ty | B.A.D. Stocker[a] | + | Aromatic compound-dependent mutant (15) |
| *S. typhi* 626Ty | B.A.D. Stocker[a] | − | Aromatic compound-dependent mutant (15) |
| *S. typhi* 619Ty | B.A.D. Stocker[a] | + | Aromatic compound-dependent mutant (15) |
| *S. typhi* 625Ty | B.A.D. Stocker[a] | − | Aromatic compound-dependent mutant (15) |
| *S. typhi* Ty21a | R. Germanier[b] | − | *galE* Mutant (11) |
| *S. dublin* | B.A.D. Stocker | + | |
| *Citrobacter* strain 5396/38 | K.H. Wong[c] | + | |

[a] Department of Medical Microbiology, Stanford University Medical School, Stanford, Calif.
[b] Swiss Serum Institute, Bern, Switzerland.
[c] Centers for Disease Control, Atlanta, Ga.

In the competitive ELISA, MAbs labeled with horseradish peroxidase by the method of Avrameas and Ternynck (1) were mixed with competing antibodies (either homologous or heterologous MAbs) before being added to antigen-coated wells. The rest of the procedures followed the standard ELISA protocol. In these competition experiments, a 1:100 dilution of the enzyme-labeled MAb was mixed with an equal volume of a 1:10 dilution of the unlabeled MAb before being added to various concentrations of solid-phase Vi antigen. The concentrations of labeled and unlabeled MAbs used were determined by prior titrations: a 1:100 dilution of the labeled MAb bound to solid-phase Vi antigen yielded an ELISA optical density (OD) of 0.45 upon substrate addition, while a 1:10 dilution of the unlabeled MAb could completely block the binding of the homologous labeled MAb and thus reduce the ELISA OD to background levels. In some experiments, unlabeled MAbs were mixed with competing monospecific rabbit antisera to O factor 9 (anti-9 O serum) and Vi (anti-Vi serum), and the binding of MAbs was tested by the indirect ELISA.

**Whole-cell radioimmunoassay.** Formalin-fixed and washed cells were adjusted to an OD at 600 nm of 1.20 for use in the whole-cell radioimmunoassay. Aliquots (0.5 ml) of the cell suspensions were dispensed into small polystyrene vials (40 by 6 mm; Sterilin Ltd., Feltham, England), and the cells were spun down in an Eppendorf centrifuge. After removal of the supernatant by suction, bacterial cells were suspended and mixed with 400 μl of MAb-containing hybridoma culture supernatant. After 2 h of incubation at 37°C, bacterial cells were washed four times with saline-Tween. Protein A (Sigma) radiolabeled with $^{125}$I (International plc, Buckinghamshire, Amersham, England) by the method of Greenwood et al. (13) to a specific activity of 800 cpm/ng was added in a 400-μl volume (containing 40 ng of protein A) to each vial; the vials were then incubated at 37°C for 2 h. After four final washes with saline-Tween, the amount of radioactivity bound to the bacterial cells was counted in a Gamma 4000 counter (Beckman Instruments, Inc., Palo Alto, Calif.). Each test was done in duplicate and with unused culture medium or 5% BSA in PBS as a control. For each bacterial strain tested, the positive/control ratio was calculated as counts per minute bound in the presence of MAb divided by counts per minute bound in the absence of MAb.

**Dot radioimmunoassay.** A 1-mg/ml Vi antigen solution (5 μl) was spotted onto each 5-mm-diameter nitrocellulose paper (0.45-μm pore diameter; Schleicher & Schuell, Dassel, Federal Republic of Germany) dot held in a well of a flat-bottomed ELISA microtiter plate. The antigen solution was allowed to dry at room temperature, and any unbound sites on the nitrocellulose paper were saturated with 5% BSA in PBS. After three washes with saline-Tween, 100 μl of hybridoma culture supernatant containing MAb was added to Vi antigen-coated dots and control dots without Vi antigen but saturated with 5% BSA in PBS. After 2 h of incubation at 37°C, and four washes with saline-Tween, 40 ng of $^{125}$I-labeled protein A (800 cpm/ng) was added to each dot for incubation at 37°C for 2 h. After four final washes with saline-Tween, the dots were counted for radioactivity as described above, and the positive/control ratio was calculated as counts per minute bound to dots coated with Vi antigen divided by counts per minute bound to control dots not coated with Vi antigen.

## RESULTS

**Serological response to Vi antigen in mice immunized with Vi antigen-containing *S. typhi* cells or purified Vi antigen.** All

TABLE 2. Serological responses of immunized BALB/c mice to purified Vi antigen

| Immunization agent | Immunization scheme | No. of mice immunized | Serological response (titer) determined by: PHA | ELISA[a] |
|---|---|---|---|---|
| *S. typhi* 620Ty whole cells[b] | Weekly for 6 wk; bled 1 wk after last injection | 3 | <1:10 | <1:100 |
| | Twice 30 days apart; bled 1 wk after last injection | 3 | <1:10 | <1:100 |
| Purified Vi antigen[c] | 1 μg once; bled 10 days after injection | 2 | 1:640–1:1,280[d] | >1:3,000 |
| | 5 μg once; bled 10 days after injection | 1 | 1:640[d] | >1:3,000 |
| | 50 μg once; bled 10 days after injection | 2 | <1:10 | <1:100 |
| | 50 μg thrice; bled 10 days after last injection | 2 | <1:10 | <1:100 |

[a] The ELISA titer was the highest dilution of serum giving an ELISA OD of 0.100.
[b] $10^8$ cells in 0.1 ml of PBS were injected intraperitoneally.
[c] Purified Vi antigen was mixed with an equal volume of Freund incomplete adjuvant and injected intraperitoneally.
[d] Atypical hemagglutination pattern; see text for details.

six mice immunized with *S. typhi* whole cells failed to yield serum antibodies to Vi antigen, as detected either by PHA or ELISA (Table 2). The three mice that had received either 1 or 5 μg of purified Vi antigen responded similarly, with ELISA titers of 1:3,000 or more. Sera from these three mice, when titrated against purified Vi antigen by PHA, failed to show typical hemagglutination patterns macroscopically; after incubation with immune mouse serum, the Vi antigen-sensitized erythrocytes neither formed a mat nor settled as a small discrete cell button. Upon microscopic examination, however, hemagglutination was observed as aggregates or clumps of erythrocytes up to a titer of 1:640 to 1:1,280 in sera from these three mice. The two mice which had been immunized similarly but with a higher dose, 50 μg per mouse, produced no detectable antibodies, even after repeated immunization.

**Production and characterization of MAbs to Vi antigen.** On day 11 after a single immunization, spleen cells from a mouse which had received 1 μg of purified Vi antigen and had produced a high titer of antibodies to Vi were fused with NSI myeloma cells. From this fusion four stable clones that produced high levels of specific Vi antibodies were eventually obtained. Their immunoglobulin classes, subclasses, and light-chain patterns were as follows: IgG2b with kappa light chains for three of the four MAbs (Vi4a, Vi5a, and Vi6a) and IgG3 with lambda light chains for the other MAb (Vi7a). Antibodies from these four hybridomas were all shown to bind staphylococcal protein A, as determined by a dot radioimmunoassay with $^{125}$I-labeled protein A.

The specificities of the MAbs were studied by a whole-cell radioimmunoassay and a competitive ELISA with commercial (Wellcome Research Laboratories, Beckenham, England) monospecific rabbit anti-Vi and anti-9 O sera. All four MAbs produced essentially the same results (Tables 3 and 4).

Using various *Salmonella* spp. with or without Vi antigen, we showed that all four MAbs not only recognized the Vi antigen in *Citrobacter* strain 5396/38 but also reacted with the Vi antigens in *S. typhi* and *S. dublin* strains. Other tested *Salmonella* species and *S. typhi* mutants that had lost the Vi antigen did not react with any of the MAbs.

In a competitive ELISA, the commercial monospecific anti-Vi antiserum but not the anti-9 O antiserum was found to block completely the binding of the MAbs to solid-phase Vi antigen, thus confirming the specificities of the MAbs.

To determine whether the epitopes recognized by the four MAbs were identical, overlapping, or physically close so that the binding of one MAb to the Vi antigen could sterically block the binding of another MAb to a different but nearby epitope, we carried out a competitive ELISA with unlabeled MAbs to compete with enzyme-labeled MAbs for binding to solid-phase Vi antigen. Results from the competitive ELISA experiments showed that unlabeled MAbs Vi4a, Vi5a, and Vi6a could completely block the binding of labeled MAb Vi4a or Vi6a. MAb Vi7a appeared to be less efficient in this regard, although complete blocking still occurred when solid-phase Vi antigen was present at a limiting concentration (0.1 μg/ml).

**Practical application of MAbs as diagnostic reagents for slide agglutination of Vi antigen-bearing bacteria.** Immunoglobulins precipitated by ammonium sulfate at 50% saturation from Vi4a-, Vi5a-, Vi6a-, and Vi7a-containing ascitic fluids had similar protein contents of 14, 14.5, 11.5, and 14.5 mg/ml, respectively. Using these ammonium sulfate-precipitated immunoglobulins for slide agglutination with cells of a Vi antigen-positive *S. typhi* strain, we found the endpoint titers to be 1:32, 1:16, 1:4, and 1:2 for the Vi4a, Vi5a, Vi6a, and Vi7a clones, respectively. Vi4a was thus selected for further testing. A total of 10 clinical blood isolates of *S. typhi*, Vi antigen-positive *S. dublin*, and *Citrobacter* strain 5396/38 were all rapidly and strongly agglutinated by ascitic fluid containing MAb Vi4a, while 21 nontyphoid *Salmonella* isolates were all negative. In addition, we also tested 18 *Escherichia coli*, 18 *Klebsiella pneumoniae*, 7 *Proteus mirabilis*, 6 *Enterobacter aerogenes*, 4 *Serratia marcescens*, 3 *Citrobacter freundii*, 5 *Acinetobacter anitratus*, 2 *Aeromonas hydrophila*, and 14 *Pseudomonas aeruginosa* strains, and they were all negative in the slide agglutination test with Vi4a-containing ascitic fluid.

TABLE 3. Whole-cell radioimmunoassay with Vi MAbs and various bacteria with or without Vi antigen

| Species | Presence (+) or absence (−) of Vi antigen | Avg[a] positive/control ratio for MAb: | | | |
|---|---|---|---|---|---|
| | | Vi4a | Vi5a | Vi6a | Vi7a |
| *S. typhi* 620Ty | + | 20.7 | 23.0 | 18.8 | 16.2 |
| *S. typhi* 626Ty | − | 0.8 | 1.0 | 0.7 | 0.6 |
| *S. typhi* 619Ty | + | 4.4 | 5.2 | 4.4 | 4.8 |
| *S. typhi* 625Ty | − | 0.8 | 1.2 | 0.8 | 0.7 |
| *S. typhi* Ty21a | − | 0.8 | 0.9 | 0.8 | 1.3 |
| *S. dublin* | + | 16.3 | 14.6 | 13.2 | 11.2 |
| *S. typhimurium* | − | 1.1 | 0.8 | 0.9 | 0.9 |
| *S. sendai* | − | 1.0 | 0.6 | 1.2 | 1.3 |
| *S. choleraesuis* | − | 0.9 | 0.8 | 0.8 | 0.8 |
| *Citrobacter* strain 5396/38 | + | 5.5 | 6.2 | 5.8 | 5.0 |
| *E. coli* | − | 0.7 | 0.9 | 1.1 | 0.8 |

[a] Average of duplicate determinations.

TABLE 4. Competition in the binding of MAb Vi4a[a] to solid-phase Vi antigen by commercial rabbit anti-Vi and anti-9 O sera

| Antibody or antiserum | Mean ELISA $OD_{492}$[b] for: | | % Inhibition |
|---|---|---|---|
| | Wells coated with 10 ng of Vi antigen | Wells coated with 5% BSA in PBS only | |
| MAb Vi4a alone | 0.211 | 0.006 | 0 |
| MAb Vi4a and rabbit anti-Vi serum | 0.012 | 0.004 | 95 |
| MAb Vi4a and rabbit anti-9 O serum | 0.227 | 0.002 | 0 |
| Rabbit anti-Vi serum alone | 0.005 | 0.003 | |

[a] Similar results were obtained with MAbs Vi5a, Vi6a, and Vi7a.
[b] Mean of triplicate determinations. $OD_{492}$, OD at 492 nm.

## DISCUSSION

Although the present study was not designed to answer the question of how mice responded to immunization with Vi antigen, it was sufficient to determine that Vi antigen present on whole bacterial cells was not a good immunogen for inducing antibodies in mice, probably because of antigenic competition. In addition, immunization of mice with Vi

antigen purified by the mild ethanol-Cetavlon method at a dose of 1 or 5 μg per mouse induced good antibody production. Even after a single immunization, mice were already producing higher titers of Vi antibodies, as assessed by the ELISA. Furthermore, the antibodies produced only microscopic hemagglutination and did not produce typical patterns of macroscopic hemagglutination with Vi antigen-sensitized cells, suggesting that the antibodies produced were of the incomplete type, most probably of the IgG class. Indeed, the four stable hybridomas obtained from fusion with spleen cells from one of the Vi antibody-producing mice all produced Vi MAbs of the IgG class.

The inability of Vi antigen-bearing *S. typhi* whole cells to induce Vi antibodies was not without precedent. The failure of whole-cell vaccines to induce protective immunity in groups A and C meningococcal meningitis has been documented (12, 28), while the purified capsular polysaccharide extracted from these bacteria conferred group-specific immunity through the induction of specific antibodies (11). Similarly, most typhoid patients did not have serum antibodies to Vi antigen, despite the fact that practically all clinical blood isolates of *S. typhi* were positive for Vi antigen (6, 8). Felix et al. (8) found that only 8 to 29% of the typhoid patients they studied (whose illness ranged from 2 to 10 weeks) had antibodies to Vi antigen, and Lanata et al. (18) detected Vi antibodies by PHA in only 38% of typhoid patients. In our study involving 44 culture-confirmed typhoid patients, only 4 had serum antibodies to Vi antigen, despite the presence of antibodies to the O and H antigens (unpublished observation).

Although several reports have described the serological response of rabbits to Vi antigens, similar measurements of antibody responses to Vi antigen were not undertaken in studies involving mice (20, 25, 27). Instead, mouse protection tests were done to assess the immunogenicity of Vi antigens. Such studies have shown that doses of 0.5 to 1.0 μg of purified Vi antigen per mouse could confer a high level of protection against challenge with Vi antigen-positive organisms. Our finding that immunization with 1 to 5 μg of purified Vi antigen stimulated good antibody production in mice seemed to support the results of previous studies. The failure to induce antibodies with a higher (50 μg) dose of Vi antigen probably reflected immune paralysis (2, 9), which has been reported to be a characteristic of the dose-dependent immune response to highly polymerized capsular polysaccharide antigens.

The Vi antibodies produced in the immunized mice were most probably of the IgG class because they did not produce typical hemagglutination patterns with Vi antigen-sensitized erythrocytes, and the resulting hybridoma MAbs obtained from one of these mice were all of the IgG class. It is of interest to note that Landy (19) also found that Vi antibodies produced in human volunteers immunized with Vi antigen were of the IgG class, and only about one-third of the vaccinees produced Vi antibodies. In our study of Vi serology in typhoid carriers by a radioimmunoassay (5), we also found that Vi antibodies in carriers were mainly of the IgG class, thus supporting the contention that the ELISA or counterimmunoelectrophoresis is a better method than PHA for serological screening of typhoid carriers.

Previous studies (reviewed in reference 22) on immunization with Vi antigen produced confusing serological results. With the availability of highly purified Vi antigen prepared by nondenaturing methods and sensitive immunoassays that can measure immunoglobulin class-specific antibodies, a more detailed study of the immune responses of human volunteers and experimental animals to immunization with Vi antigen, either in the purified form or as antigen present on bacterial whole cells, could provide the missing information concerning the kinetics of the immune response to this highly polymerized bacterial capsular polysaccharide antigen. This information would be very useful if attempts to use Vi antigen for vaccination against typhoid fever are made.

Results from the competitive ELISA showed that all four MAbs to Vi antigen reacted to either the same epitope or physically very close epitopes so that any one of these MAbs could completely block the binding of the other. Also, the finding that all four MAbs were of the IgG class suggested a restricted heterogeneity of the serum antibody response similar to that described for many polysaccharide antigens (16). An alternative explanation for this possible restricted immune response might be the monotonous regularity of the structure of Vi: a linear homopolymer of α1→4(2-deoxy-2-*N*-acetylgalacturonic acid) (14) which is highly polymerized to yield molecules of very high molecular weight.

From the specificity study (Table 3) it was clear that the four MAbs reacted with not only the Vi antigen from *Citrobacter* strain 5396/38 but also that from *S. typhi* and *S. dublin*. By using such highly specific MAbs coupled with a very sensitive radioimmunoassay, it was possible to quantitate Vi antigens such as those present on cells of different bacterial strains. By using this method, we found that *S. typhi* 620Ty, a potential candidate for a live oral typhoid vaccine, was rich in Vi antigen (Table 3), while the *galE* mutant of *S. typhi* Ty21a (10) did not possess Vi antigen.

Despite the IgG nature of the Vi MAbs, bacterial cells bearing Vi antigen were clearly agglutinated by the hybridoma-induced ascitic fluid, as demonstrated by the slide agglutination test. This Vi-agglutinating ability of the MAbs might have application in the identification of *S. typhi*. The present commercially available monospecific Vi-agglutinating serum is obtained from hyperimmunized rabbits through extensive absorption to remove agglutinins for the many common cross-reacting antigens present in other *Salmonella* species and related bacteria; rigorous testing with related bacteria must be done for each batch of serum prepared to ensure its monospecificity. Use of the present Vi-agglutinating MAbs would greatly simplify the production of such a valuable serological reagent.

Work is now in progress in our laboratory to develop further applications of these MAbs, including the detection of Vi antigen in urine from typhoid patients for early diagnosis. Previous work in this field showed only limited success and made clear the need for more specific Vi antibodies such as monoclonal antibodies. Hyperimmunized experimental animal sera to *Citrobacter* strain 5396/38 and *S. typhi* Ty2 caused an unacceptably high false-positive rate in nontyphoid patients (24). We found that our MAbs to Vi antigen could detect soluble antigens in the spent medium of Vi antigen-positive *S. typhi* strains grown in brain heart infusion broth. Also, purified Vi antigen added to normal urine at a final concentration of 1 ng/ml could be detected by these MAbs in either an indirect ELISA with rabbit anti-Vi-serum-coated plates or in a dot blot radioimmunoassay with $^{125}$I-labeled protein A. Presently, we are attempting to use these MAbs to detect Vi antigens in urine from typhoid patients.

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