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Demonstration of an antigenic protein specific for Salmonella typhi

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Summary Current studies were undertaken to determine the presence of a specific antigenic protein on the outer membrane of Salmonella typhi. Immunoblot analysis using sera from patients with fevers revealed that the 50 kD band was specifically recognized only by typhoid sera. The 50 kD band located on the outer membrane is protein by nature and is not a Vi (capsular), dH (flagellar), or O9 (somatic) antigen of S. typhi. These results indicate the usefulness of the specific antigen in the development of a serodiagnostic test for typhoid fever since antibodies of both the IgM and IgG class responses were obtained.

Typhoid fever remains as an unsolved problem especially in third world countries. There are approximately 12 million cases yearly and unless sanitary and nutritional conditions improve, it is impossible to eradicate the disease (1). Currently available methods to diagnose the disease are by isolation of Salmonella typhi from patient's blood, stool, or urine or antibody detection by means of the Widal test. Among the limitations of the culture method is low isolation rate and the value of the Widal test has been questioned with regard to its lack of specificity especially in endemic areas (2). Both methods also proved to be time-consuming, since isolation of the organism may occur between 2-8 days, while the Widal test will take 24 hours to produce results of a single specimen and 2 weeks to show a four-fold increase in titre, if any. Thus there is a need for an alternative test for typhoid that is rapid as well as specific.

Due to the localization of the outer membrane proteins (OMPs) on the surface of gram-negative bacteria (3,4), OMPs have been recently considered as important and valuable immunogens. Although cross-reactions among gram-negative OMP antigens can occur, it is important to find out if typhoid sera recognised an OMP specific for S.typhi. In this work, we demonstrate the presence of a specific OMP of S.typhi that is immunogenic in humans.

MATERIALS AND METHODS

Bacterial strain

The virulent strain S.typhi was originally isolated from a patient with typhoid fever and has been maintained in our laboratory since 1987.

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Isolation of OMPs

Partially purified outer membrane proteins of S. typhi were obtained following the methodology as described by Schnaitman (5). Briefly the bacteria was grown in nutrient broth and incubated in a shaker at 37°C for 18 h until late log phase (OD = 0.8 at 660 nm). Cells were harvested and suspended in 0.01M HEPES buffer (pH 7.4). Bacterial cells were distrupted by vortexing with glass beads (0.15 mm diameter) for 1.5 h with 1 min alternate on ice until 95 % breakage was obtained as monitored by serial gram stain. The cell lysate was aspirated and the glass beads washed with 0.01M HEPES buffer until the washings were clear. Cell debris and unbroken cells were removed by centrifugation at 5,000 x g for 15 min at 4°C. The supernatant fluid was centrifuged at 200,000 x g for 1 h to obtain cell envelopes. The cytoplasmic membrane was removed by 0.01M HEPES containing 2% Triton X-100 and was allowed to stand for 10 min at room temperature followed by centrifugation at 200,000 x g for 1 h, 4°C to pellet the insoluble outer membrane. Protein concentration of OMP was determined by the colourimetric microassay using the Bio-Rad Protein assay dye reagent (Bio-Rad, Richmond, CA) with bovine serum albumin as a standard.

SDS-PAGE and Western blots

Western blots were done essentially as described by Towbin (6). Briefly, partially purified OMPs were solubilized with SDS and resolved by SDS-PAGE using 8% acrylamide (7). The proteins were transblotted onto nitrocellulose paper for 1 h (when using a mini gel) at 30V in 25 mM Tris (pH 8.3) , 192 mM glycine and 20% methanol. The nitrocellulose paper was blocked with 3% skimmed milk dissolved in 10 mM Tris-HCl (pH 7.5) and 500 mM NaCl (buffer B) for 1 h at room temperature. The blocked nitrocellulose were cut into strips and incubated overnight with 1:200 dilution of pooled normal sera, pooled typhoid sera, sera from patients with fevers common in the region such as dengue, hepatitis, scrub typhus, paratyphi A, B and C, and monovalent sera against S.typhi antigens such as Vi, dH and O9 (Wellcome reagents). Pooled normal sera consisted of sera obtained from 5 healthy individuals while pooled typhoid sera consisted of 5 culture positive sera. The antibody treated nitrocellulose strips were washed 3 times in buffer B and incubated for 2 h with peroxidase-conjugated anti-human IgM (1:400) and IgG (1:1600), anti-rabbit IgG (1:1600) and anti-horse IgG.(1:1600). Immunoreactive bands were visualised by the addition of H_2O_2/4-chloro-1-naphthol reagent. The OMP profile resolved on SDS-PAGE gels were visualized with Coomassie blue. LPS was visualized with the periodic acid-Schiff procedure of Fairbanks et al.(8).

RESULTS

OMP preparation

The isolated OMP showed differences in electrophoretic pattern between fractions obtained during the process of purification (Fig.1). As seen, the Triton X-100 soluble fraction (inner membrane) (lane B), had more than 20 bands compared to the insoluble fraction (OM) (lane A). Most of the bands obtained differ from those observed in the OM. The OM preparation showed the presence of major and minor protein bands with the characteristic group of major proteins (porins) between 36 kD and 38 kD similar to those previously reported (9,10).

Identification of the specific antigen

Western blot procedures showed marked differences in the S. typhi - OMP antibody responses of pooled typhoid sera and pooled normal sera (Fig. 2, lanes 1 to 4). Although antibodies in the IgM and IgG class recognized almost similar bands on the OM, differences in the proteins recognized were also observed. IgM antibody in normal sera did not recognize the OMPs but the IgG class recognized at least 8 bands. By comparison to

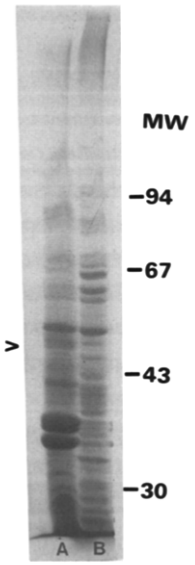

Figure 1. SDS-PAGE of S. typhi OMPs. Lanes; A , Triton X-100 insoluble fraction (outer membrane); B, Triton X-100 soluble fraction (inner membrane). Molecular size markers: phosphorylase-b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD) and lactalbumin (14.4 kD).

the normal sera, 3 bands located at 92 kD, 62 kD, and 50 kD were recognized only by typhoid sera. To determine if the antigenic bands observed were specific for S. typhi, an immunoblot comparison was carried out with sera from patients with fevers common in the

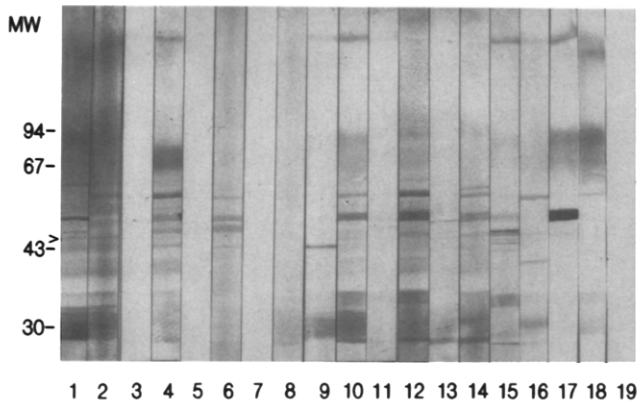

Figure 2. Western blot of various sera against S. typhi OMPs. Sera diluted 1:200 were from typhoid sera (lanes 1-2), normal sera (lanes 3-4), dengue (lanes 5-6), hepatitis (lanes 7-8), paratyphoid A (lanes 9-10), paratyphoid B (lanes 11-12), paratyphoid C (lanes 13-14), scrub typhus (lanes 15-16), anti-dH (lane 17), anti-O9 (lane 18) and anti-Vi (lane 19). With the exception of lanes 17-19, all odd numbered lanes were probed with anti-human IgM (1:400) and even numbered lanes with anti-human IgG (1:1600). For lanes 17-18, anti-rabbit IgG (1: 1600) was used, and for lane 19, anti-horse IgG (1:1600) was used.

region. Although the sera tested showed variability in their recognition of S. typhi OMPs, none cross-reacted with the 50 kD band except for typhoid sera (Fig. 2, lanes 5 to 16). With regard to its location, Fig. 1, lanes A and B, revealed that the 50 kD is located on the OM. To determine if this band is either an O9, dH or Vi antigen, commercially prepared polyclonal monovalent antisera for the 3 antigens were used. Since Vi is a capsular antigen, Anti-Vi did not recognize any bands on the OM of S. typhi. The dH and O9 antisera recognized the 52 kD and 60 kD bands respectively (Fig. 2, lanes 17 to 19), but not the 50 kD band. Analysis of the LPS content of the OM of S. typhi revealed that LPS molecules of various sizes occur in th OM. The periodic acid-Schiff stained gel did not pick up the 50 kD band (data not shown).

DISCUSSION

The purpose of our study was to determine which, if any, OMPs of S.typhi are antigenic as well as specifically recognized by typhoid sera. To determine which of these were antigenic, we used the Western blot technique and found that typhoid sera recognized both the major and several additional minor OMPs better than nonimmune sera. Reasons why healthy individuals show antibodies to S. typhi OMPs are not clear. Possibly, the S.typhi OMP shares antigenic determinants with those of other gram-negative organisms to which normal persons are usually exposed. Another is that since typhoid infection is so prevalent in Malaysia, healthy persons may have been previously exposed with S.typhi or related organisms.

Although sera from uninfected healthy individuals showed cross-reacting antibodies to several of the OMPs, it did not recognise the 92 kD, 62kD and 50 kD bands. This suggest that the antibodies present in typhoid sera had been acquired as a result of a specific infection. To determine if these antigenic bands were indeed specific for S. typhi, we further tested with sera of fevers common in the region such as dengue, hepatitis, scrub typhus, and paratyphoid fevers of A,B, and C to see if these sera cross-reacted with the 3 bands. Paratyphoid B sera recognised the 62 kD band while paratyphoid A,B, C and scrub typhus sera recognized the 92 kD band. However, all of the sera tested failed to cross-react with the 50 kD band. Similar results were obtained with sera of patients with toxoplasmosis, malaria, leptospirosis and gram-negative septicemia (data not shown). This study provided evidence that the 50 kD band is antigenic and specific for S. typhi.

Further characterization of this 50 kD band revealed that it is located on the outer membrane. When probed with sera made against the S. typhi antigens, the 50 kD band is not a Vi, dH or O9 since anti-Vi, anti-dH, and anti-O9 failed to recognize it. The 50 kD band did not pick up the periodic acid-Schiff stain suggesting that it is not a glycoprotein nor an LPS in nature.

SDS-PAGE of the OMP of S. typhi showed the presence of a small amount of this protein, and yet it seemed to be a good immunogen since pooled culture-positive sera exhibited specific IgG and IgM response to the 50 kD antigen. Being a specific antigen,

the latter could be useful for the serodiagnosis of typhoid fever in patients with fever of unknown origin. The use of a specific antigen has many advantages over the conventional methods currently used in the diagnosis of typhoid fever. Development of a rapid, specific and sensitive diagnostic test for typhoid would provide initial clinical decision making and a method to assess epidemiologically the impact of the disease. The development of such a test is currently in progress in our laboratory. Preliminary findings (unpublished data) showed 100 % correlation with over 100 S. typhi culture positive sera.

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