JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1992, p. 732–734
0095-1137/92/030732-03$02.00/0

Vol. 30, No. 3

Development of an Immunoassay Using Recombinant Maltose-Binding Protein–STa Fusions for Quantitating Antibody Responses against STa, the Heat-Stable Enterotoxin of *Escherichia coli*

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Received 12 August 1991/Accepted 4 December 1991

A set of fusion proteins containing heat-stable enterotoxin (STa) and maltose-binding protein were engineered. These molecules were readily purified and used as solid-phase antigens in an enzyme-linked immunosorbent assay to monitor anti-STa responses in mice immunized with a recombinant vaccine composed of STa and the B subunit of heat-labile enterotoxin.

Enterotoxigenic *Escherichia coli* (ETEC) is responsible for causing severe and life-threatening diarrheal diseases in humans (13) and domestic animals (7). ETEC produces either or both of the following enterotoxins: a small 19-amino-acid heat-stable enterotoxin (STa) and a large multisubunit heat-labile enterotoxin (LT) which is structurally and functionally related to cholera toxin. Recent findings have led to great interest in the use of the B subunit of cholera toxin or LT as a vaccine carrier for small antigens and epitopes (5). The B subunits exhibit the following properties: they are nontoxic, they are potent immunogens able to elicit systemic and mucosal responses following oral administration, and they can be used as purified antigens or delivered in attenuated strains of bacteria (11, 15).

Our studies have focused on the use of the B subunit of LT (LT-B) as a carrier for the genetic attachment of the small and poorly immunogenic STa to generate a putative vaccine antigen that could elicit protective antibody responses against both LT and STa. Genes encoding STa have been cloned and characterized, revealing that the toxin is initially translated as a 72-amino-acid precursor (12); an amino-terminal signal sequence is removed during export of the protein across the cytoplasmic membrane, and an internal "pro" region (amino acid residues 20 to 53), with no known function, is cleaved in the release of the 19-amino-acid carboxy-terminal toxin domain from the cell (3).

In common with others (17), we have found that antibodies against STa fail to bind to microtiter plates coated with the toxin, perhaps because of the interaction of critical regions of STa with the solid phase. Such a problem is a major obstacle to the unambiguous assay of anti-STa responses stimulated by our LT-B/STa fusions. Thus, in an attempt to circumvent this problem, we have genetically linked two STa sequences to the maltose-binding protein (MBP) of *E. coli* to generate solid-phase antigens that can be used to monitor anti-STa antibody responses.

Recombinant MBP/STa fusions were generated in the following way. Plasmid pCG806 (2), which encodes MBP (generously provided by P. Riggs, New England BioLabs, Beverly, Mass.), was digested with *Sma*I and *Pst*I, and two STa-coding sequences, excised from pJS006 and pJS007 (14) as *Sma*I-*Pst*I fragments, were ligated into the vector. Competent *E. coli* CC118 (10) cells were transformed with the ligation mixtures, and recombinant plasmids were isolated, cut with *Kpn*I, trimmed with T4 DNA polymerase, and religated. This procedure generated plasmids pMBP/ST (carrying a sequence from pJS006 and linking MBP with the carboxy-terminal toxin domain of STa [12, 14]) and pMBP/pro-ST (carrying a sequence from pJS007 and linking MBP with STa sequences which included both pro and carboxy-terminal toxin domains [12, 14]). A control, pMBP/NN (NN = nonnative), was also generated by restriction of pCG806 with *Kpn*I, trimming, and religation.

Cultures of *E. coli* CC118 carrying plasmids pMBP/NN, pMBP/ST, and pMBP/pro-ST were grown in Luria broth at 30°C, induced with 0.5 mM isopropylthiogalactopyranoside (Sigma, Poole, Dorset, United Kingdom), and grown for a further 4 h. The cells were harvested by centrifugation, and the periplasmic contents were released by treatment with EDTA and lysozyme (6). Periplasmic fractions from each strain were analyzed on a 10% polyacrylamide gel (8) (Fig. 1). The strains expressed recombinant proteins of different apparent molecular weights, corresponding to the different extensions of the carboxyl terminus of MBP. Recombinant MBPs were partially purified on cross-linked amylose resin (purchased from New England BioLabs, CP Laboratories, Bishop's Stortford, Hertfordshire, United Kingdom) (9) by following an adaptation of the manufacturer's recommendations. Proteins were eluted from columns of the resin with aliquots of a solution containing 10 mM maltose, 0.5 M NaCl, and 10 mM phosphate (pH 7.0). Samples of each fraction were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and fractions which contained recombinant MBPs were pooled (Fig. 2, lanes 1 to 4). A number of proteins from *E. coli* CC118 bound to the resin and were eluted with maltose, including the wild-type MBP (bottom band), which was common to all samples. The material derived from *E. coli* CC118(pMBP/NN) contained the modified, plasmid-encoded MBP (MBP/NN) which was of a slightly higher apparent molecular mass than the wild-type species (Fig. 2, lane 2, 40 versus 38 kDa). Fractions from *E. coli* CC118(pMBP/ST) and *E. coli* CC118(pMBP/pro-ST) contained a number of proteins of higher molecular mass than the recombinant products identified in Fig. 1 and were probably oligomers of the recombinant MBPs linked by disulfide bonds, since analysis of the same samples under

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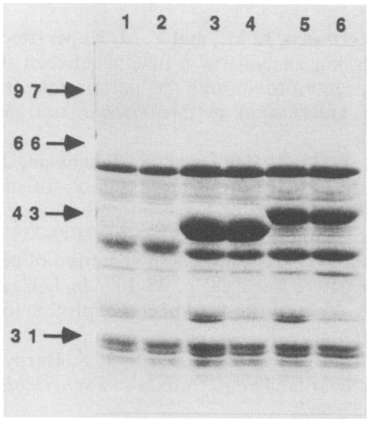

FIG. 1. Coomassie blue-stained SDS-polyacrylamide gel of periplasmic extracts from strains expressing modified MBPs. Lanes: 1 and 2, *E. coli* CC118(pMBP/NN); 3 and 4, *E. coli* CC118(pMBP/ST); 5 and 6, *E. coli* CC118(pMBP/pro-ST). Arrows to the left indicate the migration of a standard set of proteins and their molecular masses in kilodaltons.

reducing conditions resulted in the appearance of only the native and modified MBPs (data not shown). The 42-kDa product (MBP/ST) was the most abundant single component purified from the periplasm of *E. coli* CC118(pMBP/ST) (Fig. 2, lane 3), whereas *E. coli* CC118(pMBP/pro-ST) yielded a doublet of ca. 45 kDa (MBP/pro-ST) (Fig. 2, lane 4). Samples were transferred to nitrocellulose (Schleicher and Schuell, Dassel, Germany) by semidry electrotransfer (1) for Western immunoblot analysis (Fig. 2, lanes 5 to 8). An anti-STa serum raised in rabbits (generously provided by J. Clements) bound to the recombinant MBPs encoded by plasmids pMBP/ST and pMBP/pro-ST (Fig. 2, lanes 7 and 8) but failed to recognize the modified MBP encoded by pMBP/NN (Fig. 2, lane 6). Slight nonspecific binding of the antibody to a protein of approximately 90 kDa from *E. coli* CC118 was detectable (Fig. 2, lane 5), but the results demonstrate

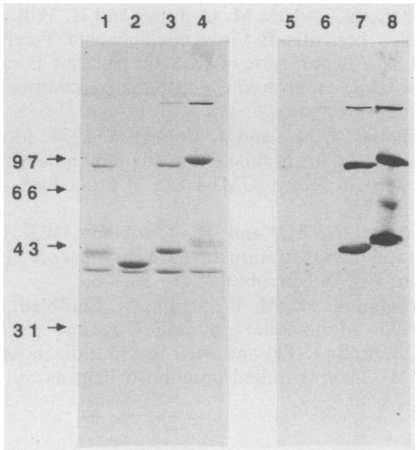

FIG. 2. Coomassie blue-stained SDS-polyacrylamide gel (lanes 1 to 4) and Western blot (lanes 5 to 8) of material purified on amylose resin columns. Lanes: 1 and 5, material from *E. coli* CC118; 2 and 6, material from *E. coli* CC118(pMBP/NN); 3 and 7, material from *E. coli* CC118(pMBP/ST); 4 and 8, material from *E. coli* CC118(pMBP/pro-ST). The Western blot was probed with polyclonal rabbit serum against STa. Arrows to the left indicate the migration of a standard set of proteins and their molecular masses in kilodaltons.

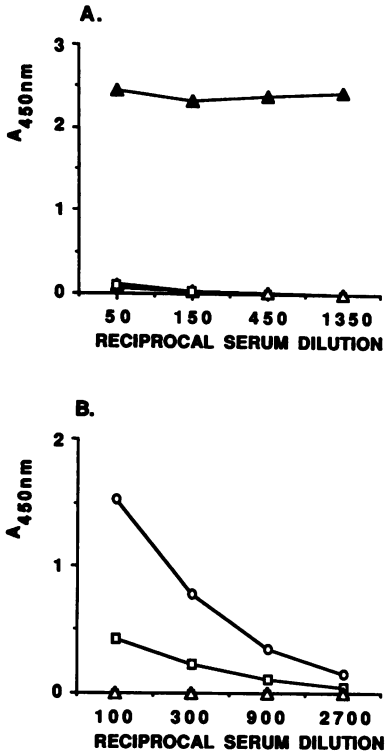

FIG. 3. Binding of polyclonal serum raised against LT-B (A) and an anti-STa monoclonal antibody, ST1:3 (B), to ELISA plates coated with GM1 ganglioside and LT-B (▲), MBP/NN (△), MBP/pro-ST (□), and MBP/ST (○). Antibody binding was detected by the addition of a goat anti-mouse antibody conjugated to horseradish peroxidase and of *o*-phenylenediamine substrate. A_{450}s were measured after 15 min.

clearly that the STa moieties in each of the fusion proteins fold sufficiently correctly to be detected by anti-STa serum.

Since it was our intention to use the recombinant MBPs in an enzyme-linked immunosorbent assay (ELISA) to assay the sera of mice immunized with a vaccine composed of STa and LT-B, we checked for serological cross-reaction between LT-B and the recombinant MBPs. The purified recombinant proteins were diluted to 1 μg/ml and applied to microtiter plates (Immulon; Dynatech, Chantilly, Va.). Various antibodies were used to check for cross-reactivity: normal mouse serum, serum against LT-B (a kind gift from R. James), and an anti-STa monoclonal antibody, ST1:3 (18) (generously provided by A. M. Svennerholm). A GM1 ELISA was used to demonstrate antibody responses against LT-B as described previously (4, 16): purified LT-B was added to GM1-coated microtiter plates at a fixed concentration (200 ng/ml), and test antisera were serially diluted. Figure 3A shows that the anti-LT-B serum had a high titer of antibodies against LT-B but showed a negligible reaction with any of the three recombinant MBPs. In addition, there was no reaction between normal mouse serum and any of the recombinant MBPs (data not shown). The reaction between the anti-STa monoclonal antibody, ST1:3, and the recombinant MBPs is shown in Fig. 3B. The monoclonal antibody failed to react with MBP/NN but recognized STa moieties in MBP/ST and MBP/pro-ST. Thus, we conclude that the adsorption of recombinant MBPs to microtiter plates displays STa moieties in the fusion proteins appropriately for

the quantitation of anti-STa antibodies and that the fusion proteins do not react with anti-LT-B immunoglobulins.

Interestingly, the reaction of ST1:3 with MBP/ST appeared to be stronger than that with MBP/pro-ST; the titer against MBP/ST was calculated to be 1,722, whereas it was 359 against MBP/pro-ST. This difference in the reactivity of ST1:3 with the two fusion proteins may have resulted from steric hindrance caused by the pro sequence (STa residues 20 to 53) (12) or from incorrect disulfide bridge formation in the STa moiety of MBP/pro-ST, indirectly affecting antibody reactivity.

Having established that the purified recombinant MPBs showed no serological cross-reaction with LT-B and that the STa moieties folded into conformations recognized by anti-STa polyclonal and monoclonal antibodies, we used the proteins in an immunoassay to monitor anti-STa responses. Female CD-1 mice (Charles Rivers, Marston, Kent, United Kingdom) were immunized with purified LT-B/STa, which stimulated a high anti-LT-B antibody titer of approximately 169,700, as assayed by a GM1 ELISA. Testing of the serum from these mice in an ELISA with the modified MBP expressed from pMBP/NN revealed a low but detectable titer of 13. It is highly likely that these antibodies were generated through contamination of the immunizing antigen with other proteins from the periplasm of *E. coli*. When the serum was diluted into wells coated with the MBP/ST construct, a significantly higher titer of 169 was observed, indicating that a specific anti-STa response had been stimulated by immunization with LT-B/STa. Considerably stronger responses were evident when the ELISA was performed with MBP/pro-ST as the solid-phase antigen; in this case the titer was 15,200. It is possible that the pro sequence of STa is more immunogenic than the carboxy-terminal toxin domain, but since the immunizing antigen contained an STa cleavage site between Met-53 and Asn-54 (3), this finding may be attributable to the loss of the latter moiety from a fraction of LT-B/STa.

We consider that the two recombinant MBP-ST fusions reported here will enable further evaluation of vaccine antigens against ETEC which express STa, and we believe that investigators interested in the potential of LT-B as a vaccine carrier for small antigens and epitopes should consider MBP-based fusions as solid-phase target antigens for immunoassays. Our recombinant MBP-ST fusions may also prove of use in modified immunodiagnostic methods for the identification of ETEC strains which produce STa (17, 18).

This work was supported by a grant from the World Health Organization Diarrhoeal Diseases Control Programme.

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