Expression of A and B Subunits of Shiga-Like Toxin II as Fusions with Glutathione S-Transferase and Their Potential for Use in Seroepidemiology

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We used the plasmid vector pGEX-2T for the expression of recombinant subunits of Shiga-like toxin II (SLT-II). The 3′ terminus of the genes that code for either the SLT-IIA or SLT-IIB subunits was genetically fused to the 3′ terminus of the gene coding for the enzyme glutathione S-transferase, which serves as a carrier in this expression system. The subunit genes were constructed synthetically by polymerase chain reaction, with appropriate restriction sites to permit in-frame downstream insertion of the genes. The resulting plasmids containing the A and B subunit genes were designated pFG1 and pFG2, respectively. Induction of Escherichia coli laboratory strains harboring pFG1 with isopropyl-β-D-thiogalactopyranoside (IPTG) yielded only small quantities of SLT-IIA fusion proteins. Since IPTG induction was lethal for cells harboring pFG2, we constructed the recombinant plasmid pFG4, which contained a subgenic fragment of slt-IIA but without the 5′ signal sequence. With this construct we were able to express very large quantities of a 33.5-kDa fusion protein, which was purified by affinity chromatography on immobilized glutathione and used as an antigen in immunoblot analysis. Rabbit serum against native SLT-II, as well as all of 12 serum samples with high neutralizing activity against SLT-II, reacted with SLT-II purified from an E. coli pFG4 expression system, whereas only 3 of 208 human serum samples with low neutralization titers and none of 54 serum samples with no SLT-II-neutralizing capability reacted. Failure of specific reactivity with the SLT-II fusion protein in the majority of human serum samples with low neutralizing activity suggests that serum factors other than immunoglobulins may be responsible for neutralizing activity in these cases. The immunoblot assay with recombinant SLT-IIB as the antigen can be recommended for use in a diagnostic setting as a simple and reliable approach to detect specific human serum antibodies to SLT-II.

Enterohemorrhagic Escherichia coli strains produce either Shiga-like toxin I (SLT-I), SLT-II, or variants of the latter. While there are enterohemorrhagic E. coli strains producing only one, there are others producing various combinations of the above toxins (20). A common structural principle of SLTs lies in the fact that they are all bipartite molecules composed of a single enzymatic A subunit and a multimer of receptor-binding B subunits (16). Recently, three types of diagnostic tests have allowed detection of either E. coli strains which produce SLTs (10, 17, 23) or human serum responses to SLTs (5, 6, 12) by assays using highly specific monoclonal antibodies, by detection of slt genes with either DNA probes or PCR, and by SLT neutralization tests. Enterohemorrhagic E. coli strains are directly responsible for a wide spectrum of diseases in both humans and animals (11). In humans, these manifestations range from asymptomatic infection to moderate diarrhea, increasing in severity to hemorrhagic colitis and even hemolytic uremic syndrome (HUS), a serious complication characterized by thrombocytopenia, hemolysis, and renal failure (12). HUS appears typically about 1 week after the onset of diarrhea. Diarrhea and HUS occur mainly in young children and also but more seldom in the elderly, consistent with the lack of immunity in these age groups (12).

There are indications of seroconversion in patients subsequent to infection; however, differentiation can be made with regard to the SLT type produced by the infectious E. coli strains. In patients suffering from HUS, increases in the titers of neutralizing antibodies against SLT-I were cited as evidence of recent infections (12). In one of several studies demonstrating SLT-I-neutralizing antibodies, 10 of 15 patients with microbiological evidence of SLT-associated HUS showed a fourfold or even greater increase in SLT-I-neutralizing antibody titers, measured in cell cultures with Vero cells (12). These results were also corroborated by NeutRELISA, which is a modification of the receptor enzyme-linked immunosorbent assay (ELISA) developed for detection of fecal SLT-I (5). Unlike patients suffering from infections with SLT-I-producing E. coli, HUS patients infected with SLT-II-producing E. coli do not show seroconversion. Caprioli et al. (6) detected high SLT-II-neutralizing activity in the serum of only 1 of 47 HUS patients, although SLT-II-producing E. coli could be cultured from stool samples of the majority of these patients. From the serological findings obtained from analyses of pooled commercial immunoglobulins to the effect that anti-SLT-I but not anti-SLT-II antibodies were present, it was assumed that the incidence of anti-SLT-II antibodies was strikingly low in the population at large (1).

We were moved by two considerations to try to develop a new assay for detecting antibodies to SLT-II. Firstly, the neutralization test is cumbersome to perform and has not been standardized for use clinically. Secondly, modern technology makes it theoretically possible to produce quantities of SLT-II antigen by recombinant means. Production of such antigen might well be of value for purposes other

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than developing a standardized test for human antibodies for use in a diagnostic setting.

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MATERIALS AND METHODS

PCR strategies for amplification of *slt-II*. The *slt-II* subunit gene and the subgenic fragment were cloned from the *slt-II* reference strain *E. coli* C600 (933W) by polymerase chain reaction (PCR) using synthetic oligonucleotides as primers (Fig. 1). The nucleotide sequences of the primers were derived from the published gene sequences of *slt-II* (20). These primers were termed GK1 and GK4, and their nucleotide sequences have been reported recently, together with those of primers GK1 and GK4, for amplifying the A-subunit gene (8). The 5' primer contained a BamHI restriction site, and the 3' primer contained an EcoRI site. For amplifying the *slt-II* subunit gene, the primers GK3 and GK4 were used as described previously (9), amplification of the subgenic fragment of *slt-II* being carried out with the primers GK6 and GKA. The PCR products were isolated by agarose gel electrophoresis, visualized by staining with ethidium bromide (8), and purified with Gene Clean (Di-anova, Hamburg, Germany).

Sequences of the 5' primers for amplifying *slt-II*/*subI* and *slt-II*/sub. The amplification of *slt-II*/*subI* and *slt-II*/sub was carried out with newly synthesized 5' primers, termed GK5 and GK6 (Fig. 1). The sequences of these primers were as follows: GK5, 5'-CCC GGA TCC TCG GAG TTG ACG-3'; GK6, 5'-CCC GGA TCC GCG GAT GTC GCT AAA GG-3'. The primers contain a BamHI restriction site (GGA TCC).

Cloning and sequencing of the PCR products. The purified DNA was digested with BamHI (GIBCO BRL, Gaithersburg, Md.) and EcoRI (GIBCO BRL) according to the manufacturer's instructions and cloned into BamHI- and EcoRI-digested pGEX-2T (Pharmacia LKB, Uppsala, Sweden). The resulting constructs were designated pFG2 and pFG4. They encode a fusion protein containing the glutathione S-transferase (GST) gene at their N terminus and the complete *slt-II* subunit (pFG2) or the subgenic fragment of *slt-II* (pFG4) at their C terminus. The fusion gene is under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible tac promoter and contains stop codons in all three reading frames at the 3' end. The cloned fragments were sequenced entirely through their 5' junctions by the dideoxy chain termination method (18, 22) with a T7 polymerase sequencing kit (Pharmacia LKB). The deduced nucleotide sequences of *slt-II* and *slt-II*/*subI* were in accord with the published sequences for *slt-II* of *E. coli* E32511 (20).

Expression and purification of recombinant fusion protein. Five-hundred-milliliter cultures of *E. coli* laboratory strain H1469 transformed with the recombinant plasmids were grown at 37°C to an *A*$_{600}$ of about 0.5 and induced to express high levels of fusion protein by the addition of IPTG to a final concentration of 1 mM. Cells were grown in the presence of IPTG for 5 h at 28°C. Cells were then harvested by centrifugation in a Sorvall centrifuge for 10 min at 3,300 × *g* and taken up in 10 ml of phosphate-buffered saline (PBS)-1% (vol/vol) Triton X-100-5 mM dithiothreitol. Bacteria were disrupted in a cell disruptor (Braun, Melsungen, Germany), cellular debris being pelleted by centrifugation at 4,400 × *g* for 10 min in a Sorvall centrifuge. The fusion proteins were present in the supernatant and were purified by the method of Smith and Corcoran (21), the incubation times being extended. Briefly, the soluble proteins were affinity purified with 500 μl of glutathione agarose beads (Sigma, Deisenhofen, Germany) by a batch procedure. After 2 h of incubation at room temperature in a rotation incubator, the glutathione beads were washed four times with PBS and collected by centrifugation at 500 × *g* for 10 s after each washing step. To elute the protein from the beads, the beads were incubated with 1 ml of 50 mM reduced glutathione (reduced) for 20 min and again collected by centrifugation at 500 × *g* for 10 s. The elution was repeated twice. Each supernatant was lyophilized overnight and diluted in 100 μl of H$_2$O. The dissolved protein was stored at −80°C. The protein concentration was determined by the method of Markwell et al. (15). The yield of recombinant protein was approximately 500 μg from a 500-ml culture.

**SLT-II-neutralization test.** Neutralizing assays and immunoblot analyses were carried out with 213 serum samples which came from blood donors and laboratory staff (aged 19 to 63 years) and with 47 serum samples from children without diarrhea or HUS (aged 8 months to 9 years) who were hospitalized for other medical reasons (3). In addition, several serum samples were from children with SLT-II producing *E. coli* O157-associated HUS. From these patients, serum samples were available from the acute phase (1 week after the onset of diarrhea) and from the postacute phase (4 to 6 weeks after the onset of diarrhea) of the disease. SLT-neutralizing antibodies were detected by the method described by Karmali et al. (12), with SLT-II preparations obtained from *E. coli* C600 (933W). Rabbit anti-SLT-I and -SLT-II antibodies have been described previously (9). For the neutralization assay, the human serum samples were diluted geometrically from 1:5 to 1:2,560. Neutralizing activities were categorized as follows: category 1, no neutralizing activity (titer of <1:5); category 2, low neutralizing activity (titer 1:5 to 1:160); category 3, high neutralizing activity (titer 1:320 to 1:2,560).

**Immunoblot analysis.** Recombinant fusion proteins were boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer containing 50 mM Tris HCl (pH 6.8), 1% (vol/vol) SDS, 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, and 0.02% (wt/vol) bromphenol blue; run on SDS-polyacrylamide (12% polyacrylamide) gels; and electroblotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) by using a Bio-Rad Mini- Protean II Dual Slab Cell. For some gels we used Tricine-SDS-polyacrylamide gel electrophoresis performed as described by Schagger and von Jagow (19). Filters were blocked in PBS-0.02% (wt/vol) sodium azide-0.05% (vol/vol) Tween 20 for 2 h and allowed to react overnight with patient or rabbit serum diluted 1:100 in PBS-Tween. Filters were washed three times for 30 min in PBS-Tween and then incubated with alkaline phosphatase-coupled goat anti-human or goat anti-rabbit immunoglobulin G (Dianova) for 2 h. Antibody binding was visualized by developing the blots with 0.01% (wt/vol) Nitro Blue Tetrazolium (Sigma) and 0.0005% (wt/vol) 5-bromo-4-chloro-3-indolyolphosphate (Sigma) dissolved in 2% (vol/vol) diethanolamine (pH 9.8) (Merck, Darmstadt, Germany).

RESULTS

**PCR amplification and cloning of *slt-IIA* and *slt-IIIb* genes.** Using the primers depicted in Fig. 1, we amplified the *slt-IIA* and *slt-IIIb* subunit genes by PCR, as described previously (8), and in addition subgenic fragments of these genes. Figure 2 shows PCR amplification products of *slt-IIA* (lane 1)
and slt-II (lane 3) that were digested with BamHI and EcoRI and cloned into BamHI- and EcoRI-digested pGEX-2T. The recombinant plasmids bearing the individual subunit genes were designated pFG1 (pGEX-2T plus slt-IIA) and pFG2 (pGEX-2T plus slt-IIIB). Cleavage of these constructs with BamHI and EcoRI showed DNA fragments of the expected sizes, 950 bp for slt-IIA (Fig. 2, lane 2) and 265 bp for slt-IIIB (lane 4). Moreover, subgenic fragments were amplified and cloned, as described above, by applying primers GK5 and GK2 to amplify slt-IIA/subl and GK6 and GK4 to amplify slt-IIIB/subl. These constructs were duly termed pFG3 (pGEX-2T plus slt-IIA/subl) and pFG4 (pGEX-2T plus slt-IIIB/subl).

Expression of fusion proteins. E. coli H1469 clones transformed with the above-mentioned plasmids or with pGEX-2T were grown to an A₆₀₀ of 0.5 and subsequently induced with IPTG to express either the GST or the recombinant fusion proteins. The kinetics of IPTG-induced protein expression are demonstrated in Fig. 3. Figure 3A, lanes 5 to 7, shows expression of GST (27.5 kDa) 1, 2, and 3 h after induction, and for comparative purposes, Fig. 3B, lanes 5 to 7, shows expression of GST + SLT-IIA (59.5 kDa). As negative controls, we employed whole-cell lysates of noninduced cells, harvested at the same time as the induced cells. There was no expression of foreign protein apparent (lanes 2 to 4 in Fig. 3A and B). When these IPTG experiments were performed on E. coli H1469 harboring the plasmid pFG2 containing the whole slt-IIIB subunit gene, the induction caused cell lysis, which resulted in a rapid decrease in A₆₀₀ from 0.5 to 0.1 within 60 min. Consequently, after centrifugation no bacterial pellets were obtainable. Therefore we constructed subgenic fragments of the slt-IIIB subunit gene in order to gain the fusion protein required. We used the primers GK6 and GK4, whose location within the slt-IIIB gene is shown in Fig. 1. This approach proved successful, as shown in Fig. 4, lanes 5 and 6. Bacteria harboring the plasmid pFG4 were able to synthesize a 33.5-kDa protein in large quantities, which was the expected molecular mass of the fusion protein consisting of GST and SLT-IIIB/SUBL.

FIG. 1. Scheme of the slt-II operon and the primers used to amplify toxin subunit genes and subgenic fragments. Primers GK1 and GK2 were used to amplify the whole slt-IIA gene, and primers GK3 and GK4 were used to amplify the slt-IIIB gene. Amplification of slt-IIA/subl was carried out with primers GK5 and GK2, slt-IIIB/subl being amplified with the primers GK6 and GK4. SD, Shine-Dalgarno sequence; LS, leader sequence. 

FIG. 2. Separation of amplified slt-II toxin gene subunits and restriction endonuclease digestions of pFG1 and pFG2 by submarine agarose gel electrophoresis. Lanes 1 and 3, PCR products of slt-IIA appearing at 950 bp and of slt-IIIB appearing at 265 bp, respectively, both indicated with arrows; lanes 2 and 4, BamHI- and EcoRI-restricted pFG1 and pFG2, with the vector pGEX-2T at 4.9 kb (arrow) and the cloned genes slt-IIA (lane 2) and slt-IIIB (lane 4), with their expected sizes. Lanes M contain a 1-kb DNA ladder (GIBCO BRL).

FIG. 3. Coomassie blue-stained SDS-PAGE gel showing whole-cell lysates of induced and noninduced E. coli H1469 harboring pGEX-2T (A) and pFG1 (B). (A) Lane 1, E. coli H1469 at an A₆₀₀ of 0.5; lanes 2 to 4, noninduced cells after 1, 2, and 3 h, respectively, showing no expression of foreign proteins; lanes 5 to 7, induced cells 1, 2, and 3 h after induction, respectively, showing the GST at the expected molecular mass of 27.5 kDa (arrow). Lane M contains molecular mass markers (Pharmacia low range). (B) Lane 1, E. coli H1469 at an A₆₀₀ of 0.5; lanes 2 to 4, noninduced cells after 1, 2, and 3 h, respectively, showing no expression of foreign proteins; lanes 5 to 7, induced cells 1, 2, and 3 h after induction, respectively, showing the GST + SLT-IIA fusion protein at the expected molecular mass of 59.5 kDa (arrow).
This protein could not be detected on SDS gels with noninduced cells. We also employed this strategy of constructing a fusion protein without the leader sequence for the A subunit using primers GK5 and GK2. We were unable to further increase the yield over that obtained with cells harboring the plasmid pFG1, which contains the whole A-subunit gene.

**Purification of recombinant SLT-II/ SUB1.** The recombinant proteins could be purified by affinity chromatography of disrupted cells on immobilized glutathione. The purified 33.5-kDa GST + SLT-II/SUB1 protein is shown in Fig. 5, lane 3. In order to remove the GST carrier from the fusion protein, cleavage was carried out with thrombin. As shown in Fig. 5, lane 4, this approach yielded a band at 6 kDa, the expected size of the SLT-II/SUB1 fusion protein. Since the major part of the fusion protein could not be cleaved under these circumstances, the yield of recombinant SLT-II/SUB1 was very low.

**Reactivity of rabbit and human sera with the fusion protein.** We used the SLT-II fusion protein purified from the E. coli pFG4 expression system as the antigen for immunoblot analysis. GST from E. coli transformed with the pGEX-2T vector and purified by affinity chromatography was applied as a control. These antigens were separated by SDS-PAGE and either stained or transferred to nitrocellulose for use in subsequent immunoblots. Figure 6A shows a stained gel, with the GST in lane 1 and the SLT-II fusion protein in lane 2. With rabbit serum, reactivity occurred with the SLT-II fusion protein (Fig. 6B, lane 2); however, lane 1 does not reveal any reactivity at all, meaning that the response is indeed directed against the GST protein and not against the GST. Preimmune serum showed no reactivity, while rabbit anti-SLT-I hyperimmune serum displayed a weak but visible band (not shown). Shown in Fig. 6C is an immunoblot analysis of a human serum sample with high SLT-II-neutralizing activity (titer, 1:640), which reveals a high degree of reactivity and specificity.

**Comparison of the SLT-II cytotoxicity neutralization assay and immunoblot reactivity of human serum.** Next we tested a total of 274 human serum samples by neutralization assay and immunoblot analysis to establish a correlation between these tests. These serum samples, originating from healthy adults, healthy children, and HUS patients, were duly categorized according to their neutralizing activity as having no measurable neutralization activity, low neutralizing titers, or high neutralizing titers. Table 1 shows the number of serum samples that fall into these three categories and indicates the reactivity of the serum samples in the immunoblot analysis with the recombinant SLT-II as the antigen. Obviously, as shown in Table 1, the titers in the neutralization assay are either low (titers of 1:5 to 1:40) or very high (titers of >1:320) for 75.9 and 4.4% of the serum samples tested, respectively. None of the samples tested yielded a titer between 1:80 and 1:320. A total of 19.7% had no neutralizing activity (titer of <1:5). All serum samples with high neutralizing activity reacted in the immunoblot analysis (Table 1). Immunoblot strips of all 10 of the serum samples with high neutralizing activity from the blood donor group are depicted in Fig. 7A.

As shown in Table 1, none of 54 serum samples without
neutralizing activity and only 3 of 208 serum samples with low neutralizing activity reacted in the immunoblot test. The two blood donor serum samples from the group with low neutralization activity, together with eight representative nonreacting serum samples, are shown in Fig. 7B.

Immunoblot analysis of serum samples from acute- and convalescent-phase HUS patients. Acute- and convalescent-phase serum samples were available from seven patients with SLT-II-associated HUS. Paired serum samples from each patient were analyzed by neutralization assay and by an immunoblot test with the recombinant SLT-IIB as the antigen. The data are shown in Table 1. In only one of the seven patients with SLT-II-associated HUS did we find a fourfold rise in SLT-II-neutralizing activity titer. Here the titer increased from 1:40 to 1:640. In the remaining convalescent-phase serum samples, no changes in the neutralization titers were observed in comparison with the acute-phase serum samples. As shown in Table 1, by immunoblot analysis only the convalescent-phase serum samples with high neutralizing activity and none of the acute-phase serum samples yielded reactivity.

DISCUSSION

SLT-producing E. coli may cause a wide spectrum of illnesses encompassing mild to moderate diarrhea, hemorrhagic colitis, and HUS, and there is also now substantial evidence that these pathogens may well be a causal factor for asymptomatic infections (11). Moreover, not all patients suffering from diarrhea develop HUS. One of the most puzzling questions in the clinical microbiological laboratory after the culture of these pathogens from stool samples is whether symptoms will develop, and if so, whether they will then lead to the life-threatening complications of HUS. From animal studies, there is evidence that SLT-specific antibodies provide protection (14), but evidence of such protection for humans is lacking. The main reason for this lies in the banal fact that epidemiological studies of humans have not been comprehensive, the neutralization tests not being standardized at all. For an initial approach to neutralization assays, we have used the pGEX-2T expression system to produce SLT-II antigen in large quantities by recombinant means. As we were able to successfully fuse a portion of the gene for SLT-IIB, our results also showed the limitations of this technique. Such restrictions included problems with the expression of the A subunit in sufficient quantities, as well as cell death caused by lysis after IPTG induction of E. coli laboratory strain H1469 harboring the recombinant plasmid pFG2, which contained the complete B-subunit gene.

We have shown that expression of fusion proteins in large quantities was possible only after we had manipulated the slit-II gene by deleting the leader sequence. The reason for this phenomenon is unknown and requires further investigation. Moreover, we encountered difficulties with thrombin cleavage and solubilization of the expressed B subunit. However, we managed to purify the SLT-IIB fusion protein from crude bacterial lysates of the E. coli pFG4 expression system by affinity chromatography on immobilized glutathione.

An assessment of the correlation between the biological SLT-II-neutralization assay and the reactivity of human serum samples with the fusion protein by immunoblot analysis revealed that all 12 human serum samples with high titers of neutralizing activity reacted in the immunoblot, whereas no reactivity was seen in 54 serum samples with no neutralizing activity. No good correlation was to be observed in a comparison between neutralization assays and immunoblot reactivity of the 208 serum samples with low SLT-II-neutralizing activity. This discrepancy might be explained in two ways. Firstly, immunoblot analysis is less sensitive than the biological assay. Secondly, factors other than immunoglobulins may cause neutralizing activity. In this context, Bitzan et al. (4) reported that low SLT-II-neutralizing activity seen in all of their serum samples from HUS patients but also in samples from healthy controls was retained after selective removal of immunoglobulins. These authors suggested that low neutralizing activity is due to serum factors other than immunoglobulins. Therefore, the diagnostic value of SLT-II neutralization assays seems to be limited, and tests with SLT-II as the antigen could differentiate between sera with and without SLT-II antibodies.

Production of SLT-II antigen by recombinant means offers

<table>
<thead>
<tr>
<th>Source of samplesa</th>
<th>No. of samples (no. of IB-reactive samples) with NA titerb</th>
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<tbody>
<tr>
<td></td>
<td>&lt;1:5</td>
</tr>
<tr>
<td>Blood donors and laboratory staff (n = 213)</td>
<td>35 (0)</td>
</tr>
<tr>
<td>Healthy children (n = 47)</td>
<td>15 (0)</td>
</tr>
<tr>
<td>HUS patients</td>
<td></td>
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<tr>
<td>Acute phase (n = 7)</td>
<td>2 (0)</td>
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<tr>
<td>Convalescent phase (n = 7)</td>
<td>2 (0)</td>
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<tr>
<td>Total</td>
<td>54 (0)</td>
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a Acute- and convalescent-phase serum samples are from the same patients.
b NA, neutralization assay; IB, immunoblot.

FIG. 7. Representative immunoblots of human serum samples with the fusion protein as the antigen. Shown are 10 immunoblot strips incubated with human serum samples from a total of 213 blood donors, containing high titers of neutralizing activity (A), and incubated with the 2 human serum samples with low neutralizing activity and positive immunoblot reactions from the blood donor group and with 8 representative nonreacting serum samples (B).
EXPRESSION OF SLT-IIA AND SLT-IIB AS FUSIONS WITH GST

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