Stable Expression and Characterization of Monomeric and Dimeric Recombinant Hybrid-IgG/IgA Immunoglobulins Specific for Shiga Toxin

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Antigen-specific immunoglobulin A (IgA) may be useful for preventing infectious diseases through passive immunization on the mucosal surface. We previously established mouse IgA and IgG monoclonal antibodies (mAbs) specific for the binding subunit of Shiga toxin 1 (Stx1B). We also developed a recombinant hybrid-IgG/IgA, in which variable regions from the IgG mAb were present. The binding activity of recombinant hybrid-IgG/IgA was verified by transient expression. Aiming at a constant supply, we established Chinese hamster ovary cells stably expressing monomeric or dimeric hybrid-IgG/IgA. The cDNAs encoding heavy and light chains were co-expressed for the monomeric hybrid-IgG/IgA, while those encoding heavy, light, and joining chains were co-expressed for the dimeric one. Serum-free culture supernatants of the cloned transfecants were subjected to size-exclusion chromatography. The elution patterns showed that the binding to immobilized Stx1B and the immunoblot signals of assembled immunoglobulins were correlated. In the transfectant for the dimeric hybrid-IgG/IgA, both monomers and dimers were observed. Size-exclusion chromatography enabled us to prepare a sample of the dimeric hybrid-IgG/IgA devoid of the monomeric one. The monomeric and dimeric forms of hybrid-IgG/IgA were prepared from the respective transfecants to examine the neutralization of Stx1. After pretreatment with monomeric or dimeric hybrid-IgG/IgA, the cytotoxicity of Stx1 toward Vero cells was abolished. Furthermore, the dimeric form was more than 10-fold more effective than the monomeric one in terms of toxin neutralization. These results suggest that the tetravalent feature of the binding sites of the dimeric hybrid-IgG/IgA contributes to the efficacy of toxin neutralization.

Key words recombinant immunoglobulin A; Shiga toxin; neutralization; dimer

Shiga toxin 1 (Stx1) is a virulence factor of enterohaemorrhagic Escherichia coli (EHEC) strains such as O157:H7 and Shigella dysenteriae.1,2) It comprises one A subunit and five B subunits.3) After binding through B subunits (Stx1B), which are cell-binding subunits, Stx1 holotoxins are endocytosed.4) The A subunit, which is translocated into the cytoplasm, induces cytotoxicity through dysfunction of ribosomes and inhibition of protein synthesis.5,6)

Immunoglobulin A (IgA) plays a role in the humoral arm of the adaptive immunity on the mucosal surface.6) It is produced by B cells after class switch of heavy (H) chains from IgM to IgA. IgA-producing B cells also produce joining (J) chains that covalently join IgA monomers to form dimeric IgA (dIgA).7) On the mucosal epithelium, the dimeric IgA is transported by polymeric immunoglobulin receptors (pIgR) from the basolateral to the apical side. Dimer formation is required for the binding to pIgR.8) On the mucosal surface, pIgR is cleaved by proteases, and the complex of dIgA and the ectodomain of pIgR is secreted onto the mucosal surface. The ectodomain of pIgR is termed a secretory component (SC) in a secretory IgA (SlgA). SlgA prevents invasion by pathogens and their virulence factors. This function of SlgA is termed immune exclusion.9) In addition, oral access to SlgA, such as through the mother’s milk, also protects infants from pathogens. This route contributes to the establishment of passive immunity due to preformed SlgA.

To achieve oral passive immunity against Stx1B, we obtained a mouse IgA monoclonal antibody (mAb) against Stx1B, termed G2G7, by intranasal immunization.9,10) We also produced an IgG1 mAb against Stx1B, termed D11C6.11) On testing of the binding of Stx1B to Burkitt’s lymphoma Ramos cells, we found that the IgG1 mAb but not the IgA one completely inhibited the binding of Stx1B to the target cells.11) To obtain an IgA mAb with stronger biological activity, we constructed a recombinant H chain. Thus, the H chain variable region and the C11 domain are from D11C6 (IgG1), while the C12 and C13 domains from G2G7 (IgA). We transiently expressed this hybrid H chain, the light (L) chain from D11C6 and the J chain obtained from G2G7 in COS-1 cells (IgG1). We found that the IgG1 mAb but not the IgA one completely inhibited the binding of Stx1B to the target cells.11) To obtain an IgA mAb with stronger biological activity, we constructed a recombinant H chain. Thus, the H chain variable region and the C11 domain are from D11C6 (IgG1), while the C12 and C13 domains from G2G7 (IgA).

MATERIALS AND METHODS

Reagents Shiga toxin 1 holotoxin (Stx1) and the recombinant purified B subunit of Stx1 (Stx1B) were prepared as described previously.11,13) Stx1B-specific mouse mAb G2G7 (IgA, k) and mouse mAb D11C6 (IgG1, k) were prepared as described previously.11,10) The cDNAs encoding the Stx1B-specific hybrid-IgG/IgA H chain, IgG1-associated L chain and J chain were prepared by subcloning into the pcDNA3.1(+) Zeo expression vec-
 tors (Life Technologies; Carlsbad, CA, U.S.A.), respectively, as described previously.12) Kanamycin sulfate, hygromycin B and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); Dulbecco's modified Eagle's medium (DMEM), Ham's F12 (F12), and medium 199 (M199) from Nissui Pharmaceuticals (Tokyo, Japan); and CD CHO-A medium, Opti-MEM I medium and zeocin from Life Technologies.

The concentration and dilution (in phosphate-buffered saline, PBS) was concentrated to 1 mL by means of Vivaspin 20-100K (GE Healthcare, Piscataway, NJ, U.S.A.).

Subconfluent conditions, the medium was replaced with DMEM/F12 in the presence of appropriate antibiotics. Under subconfluent conditions, the medium was replaced with serum-free DMEM/F12 in the presence of appropriate antibiotics. Under subconfluent conditions, the medium was replaced with serum-free DMEM/F12 in the presence of appropriate antibiotics. Under subconfluent conditions, the medium was replaced with serum-free DMEM/F12 in the presence of appropriate antibiotics.

Stable Expression of Hybrid-IgG/IgA CHO-K1 cells were transfected with the plasmid expression vectors for the hybrid-IgG/IgA H and L chains (monomeric IgA), or those for the hybrid-IgG/IgA H and L chains (dimeric IgA) by means of FuGENE 6. A total 1 µg of plasmid DNA was used to transfect subconfluent CHO-K1 cells in 1 mL of Opti-MEM in the wells of a 12-well plate ( Falcon® 353043; Corning Inc., Corning, NY, U.S.A.). Forty-eight hours after transfection, cells were selected in the presence of appropriate antibiotics in 10% FBS-DMEM/F12. The concentrations of antibiotics were as follows: 800 µg/mL G418, 400 units/mL hygromycin B and 1000 µg/mL zeocin. The cells producing monomeric or dimeric IgA specific for Stx1B were cloned by limiting dilution in 10% FBS-DMEM/F12 in the presence of appropriate antibiotics.

Gel Filtration The resulting cloned CHO-K1 cells that produced recombinant antibodies were cultured in 10% FBS-DMEM/F12 in the presence of appropriate antibiotics. Under subconfluent conditions, the medium was replaced with serum-free CHO-A medium, and culture supernatants were harvested after 3 d. Each culture supernatant (15–30 mL) was concentrated to 1 mL by means of Vivasin 20-100K (applicable volume 20 mL, exclusion molecular weight 100000). The concentration and dilution (in phosphate-buffered saline containing 0.02% Na2SO4) procedures were repeated four times to remove proteins of less than 100 kDa. Each concentrated sample (1 mL) was separated on a column of Sephacryl S-300 (1.5 cm × 40 cm) equilibrated with PBS-Na2SO4. Fractions (1 mL each) were collected to monitor OD580 (nm) and binding activity toward immobilized Stx1B.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting We carried out SDS-PAGE (non-reducing 7.5% gel and reducing 12% gel under Laemmli’s conditions) and then immunoblotting as described.12) Hybrid-IgG/IgA was detected by 1 h incubation with HRP-goat anti-mouse IgA (1 : 1000). The signal representing the H chain was enzymatically detected using a chemiluminescence reagent (West Pico; Thermo Scientific Pierce, Rockford, IL, U.S.A.). As molecular weight standards, Precision Plus Protein WesternC STDs with StrepTactin-HRP (Bio-Rad; Hercules, CA, U.S.A.) and MagicMark XP Western Protein Standard (Life Technologies) were used. The bands were directly visualized by means of chemiluminescence.

Enzyme-Linked Immunosorbent Assay (ELISA) The binding of the hybrid-IgG/IgA to immobilized Stx1B was detected with HRP-goat anti-mouse IgA by means of ELISA as described previously.23) To quantitate total hybrid-IgG/IgA in samples, sandwich ELISA was performed.23) In this case, immobilized rabbit anti-mouse IgA was used to capture antibodies and HRP-rabbit anti-mouse IgA to detect the captured hybrid-IgG/IgA.

Toxin Neutralization Assay Appropriate fractions containing hybrid-IgG/IgA were pooled and the buffer was changed to sterile PBS by means of a Vivasin 2-100K to remove NaN3, followed by sterilization by membrane filtration. The concentrations of monoclonal antibodies (mAbs) were determined by sandwich ELISA as described previously.10,11) Vero cells were plated at 2 × 10⁵ cells/100 µL of 10% FBS-M199 in the wells of a 96-well plate ( Falcon® 353072, Corning) and then cultured for 17 h. Stx1 (10 pg) and an antibody (varying amount) were mixed in 1 mL of 10% FBS-M199, followed by incubation for 1 h at 37°C. After replacing the medium with the mixture of Stx1 and an antibody, Vero cells were further cultured for 48 h. Cell viability was measured by means of a colorimetric assay using the Cell Counting Kit-8 as described.11) Viability was defined as the percentage of the control level (without toxin exposure).
molecular mass between 150 and 250 kDa (arrow, Fig. 1B), peaked at fraction Nos. 39 to 41. There were several bands with lower molecular masses that appeared in parallel with the band of molecular mass between 150 and 250 kDa. This may represent incomplete assembly of the IgA monomer or partial degradation products.

**Stable Expression of a Dimeric Hybrid-IgG/IgA against Stx1B and Its Separation by Gel Filtration**

CHO-K1 cells were co-transfected with the vector construct harboring the hybrid-IgG/IgA H chain, that harboring the D11C6-associated L chain and that harboring the J chain. Through selection with G418, hygromycin B and zeocin followed by limiting dilution cultures, we obtained 86 clones. We selected clone #2, which exhibited the strongest binding to immobilized Stx1B.

We prepared a 30 mL of serum-free culture supernatant of clone #2 (yield: 0.6–1.2 µg/mL of IgA), followed by concentration to 1 mL and then separation by gel filtration on the same Sephacryl S-300 column as that used for the separation of the monomeric hybrid-IgG/IgA. The elution pattern was monitored at OD280 nm and the binding to immobilized Stx1B by ELISA (Fig. 2A). Two peaks of binding were observed around fraction Nos. 36 and 41, respectively. We then analyzed each fraction (Nos. 35 to 41) by means of SDS-PAGE (non-reducing conditions) and immunoblotting using anti-α chain antibodies (Fig. 2B). Fraction Nos. 35 and 36 gave a band larger than 250 kDa (Fig. 2B, left arrow), while fraction Nos. 40 and 41 gave a band of molecular mass between 150 and 250 kDa (Fig. 2B, right arrow). Before gel filtration, these two bands were observed (data not shown). The results suggested that the former fractions represent the dimeric hybrid-IgG/IgA and the latter ones the monomeric hybrid-IgG/IgA.

**Characterization of the Hybrid-IgG/IgA Heavy Chain**

A supernatant of clone #3 (stably expressing H and L chains) was subjected to gel filtration to obtain a monomeric IgG/IgA (fractions 37–43). A supernatant of clone #2 (stably expressing H, L and J chains) was subjected to gel filtration to obtain a dimeric one (fractions 33–36). After concentration by means of a Vivaspin, the pooled fractions containing the dimeric (Fig. 3, lane 1) or monomeric (lane 2) hybrid-IgG/IgA were subjected to SDS-PAGE and immunoblotting under reducing conditions (Fig. 3). The recoveries of IgA from culture supernatant to the pooled fractions were 38.5% and 16.3% for the monomeric and the dimeric one, respectively. The hybrid-IgG/IgA H chains electrophoresed to a position corresponding to a relative molecular mass of 50 to 60 kDa (Fig. 3, left arrowhead). This is slightly smaller than that of mouse IgA myeloma TEPC 15 (lane 3). This may be due to the difference in domain composition, the hybrid-IgG/IgA H chain containing VH, Cγ1, Ca2 and Ca3, and the IgA H chain containing VH, Cα1, Ca2 and Ca3. The specificity for the α chain was verified by the absence of signals from mouse IgGl myeloma MOPC 21 (lane 4).

**Binding of Monomeric and Dimeric IgG/IgA to Immunobilized Stx1B**

Varying concentrations of dimeric or monomeric hybrid IgG/IgA in the Sephacryl S-300-pooled fractions were allowed to bind to immunobilized Stx1B. The dimers appeared to bind a little better than the monomers did. However, the dose–response curves in response to the total IgA concentrations were situated close to each other (Fig. 4).
An IgA myeloma TEPC 15 did not bind to Stx1B. The hybrid IgG/IgA did not bind to the wells without immobilized Stx1B.

**Toxin Neutralization by the Monomeric and Dimeric Hybrid-IgG/IgA**

In each hybrid-IgG/IgA sample, the concentration of assembled immunoglobulins was determined by sandwich ELISA. Thus, the ELISA was performed using a format whereby immunoglobulins with H chains were captured and only those with L chains can give rise to signals.

First, we examined whether the cytotoxicity to Vero cells on treatment with 10 pg/mL of Stx1 could be blocked by 1 µg/mL of an antibody (Fig. 5A). Upon 48 h cell culture with Stx1, a 50% reduction in the viability was observed with an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-like assay that reflects reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H)-dependent cellular oxidoreductase activity. An Stx1B-specific IgA mAb, G2G7, did not significantly improve the viability, as expected. On the other hand, an Stx1B-specific IgG mAb, D11C6, completely inhibited the cytotoxicity, as also expected. Since the hybrid-IgG/IgA utilizes the variable regions of D11C6, toxin neutralization activity was expected. Both the monomeric and dimeric hybrid-IgG/IgA inhibited cytotoxicity toward Vero cells.

Second, we varied concentrations of the hybrid-IgG/IgA to evaluate the relative efficacy (Fig. 5B). For the monomeric one, complete inhibition at 1 µg/mL but incomplete inhibition at 0.1 µg/mL were observed. In contrast, complete inhibition was seen at 0.01 to 0.1 µg/mL for the dimeric one, but it was not effective at 0.001 µg/mL. These results suggested that the dimeric hybrid-IgG/IgA is more than 10-times more effective compared with the monomeric one on a weight basis.
DISCUSSION

The major objective of this study is the production of cell lines expressing monomeric or dimeric recombinant IgA against Stx1B to test their toxin neutralizing activity. To obtain a sufficient and constant supply of the recombinant IgA against Stx1B, we stably expressed immunoglobulin genes against Stx1B, we stably expressed immunoglobulin genes in CHO-K1 cells. The hybrid-IgG/IgA utilizes variable regions, both H and L chains, from an IgG1 mAb that exhibits remarkable toxin neutralization activity. Because the H chain constant region was from an IgA mAb, it can be dimerized through a J chain.53 When co-expressed with J chains in CHO-K1 cells, the dimeric hybrid-IgG/IgA was observed along with the monomeric one. Thus, we needed to use size-exclusion chromatography to prepare a dimeric hybrid-IgG/IgA sample that is devoid of monomeric one.

The CHO-K1 cells transfected with H and L chains only produced monomers without dimers as revealed by immunoblotting analysis after SDS-PAGE under non-reducing conditions (Fig. 1). In contrast, CHO-K1 cells transfected with H, L and J chains produced monomers and dimers, but the dimers were isolated by size-exclusion chromatography essentially being devoid of monomers (Fig. 2). Taking advantage of these two cell lines, we could assess the biological activity of the dimeric hybrid-IgG/IgA in comparison with those of the monomeric one.

The dimers devoid of monomers were able to neutralize toxin activity with more than tenfold efficacy on a weight basis. In contrast, it appears that only a few difference between monomers and dimers in the binding activity to immobilized Stx1B, when one compares the result of each fraction on gel filtration (Figs. 2A, B). To confirm this, dose–response curves were compared between the monomer and dimer preparations in the ability of binding to immobilized Stx1B. The binding of dimers appeared to be a little more efficient, but the difference was only a small one. It may be that the dimeric IgG/IgA with tetravalent binding sites is more effective in the lattice formation with Stx1 in solution. The tetravalent nature of binding sites may add only a small effect to the binding of antibodies to immobilized antigens on an ELISA plate.

Upon transient expression of the dimeric hybrid-IgG/IgA in COS-1 cells, the relative molecular mass of the hybrid-IgG/IgA H chain was smaller than that of IgA myeloma TEPC 15.12) This was also observed in the present system using CHO-K1 cells.

We did not examine the toxin neutralization activity of the sample from transiently expressed COS-1 cells because we could not obtain sufficient amounts of antibodies. Recently, we succeeded in producing a dimeric hybrid-IgG/IgA in a plant system involving Arabidopsis thaliana.14) In this plant, there are also monomers, and several incomplete IgA and fragments. Although plantibodies are able to neutralize toxins, we could not determine the relative efficacy of intact dIgA as to the toxin neutralization. In the present study, we directly demonstrated that the dimeric form of IgA produced in CHO-K1 cells efficiently neutralizes Stx1.

To prevent infectious diseases, a vaccine strategy that leads to the production of IgA on the mucosal surface is a possibility. In this case, one needs an efficient and safe adjuvant to overcome peripheral tolerance for the initiation of immune responses against given antigens. Furthermore, probably due to the abundant commensal bacteria on the gut mucosal surface, it is not straightforward to produce SlgA specific for target antigens. Thus, many IgA may be directed to commensal bacterial species rather than specific antigens of which neutralization is required. Preformed SlgA against pathogens will become a valuable strategy in place of vaccination.

SlgA are produced through transepithelial transport of dIgA in vivo.50 That is, dIgA is the product of B cells whereas SC is the product of epithelial cells. In addition, the expression of the J chain together with the H and L chains is essential for SlgA because monomeric IgA cannot form SlgA.6,15) Some earlier studies demonstrated that SlgA could be produced when SC was co-expressed in CHO cells, however, the efficiency of the formation of SlgA was not clearly demonstrated.16) Alternatively, in vitro reconstitution is another possibility for obtaining SlgA.17) In either case, the production of dIgA of enough amount and with reasonable purity is essential to produce SlgA and to test its effect in vivo. We are currently in progress to establish protein purification scheme for the dimeric IgG/IgA.

In conclusion, we stably expressed dimeric IgG/IgA specific for Stx1B in CHO-K1 cells. The dimers, separated by size-exclusion chromatography, could efficiently neutralize toxin activity. The dimers will be useful for the preparation of SlgA. SlgA produced by cultured animal cells will serve as useful standards for the SlgA produced by plants we have developed.

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