Shiga toxin-induced apoptosis is more efficiently inhibited by dimeric recombinant hybrid-IgG/IgA immunoglobulins than by the parental IgG monoclonal antibodies

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Abbreviations: CHO, Chinese hamster ovary; J chain, joining chain; SIgA, secretory IgA; Stx1, Shiga toxin 1; Stx1B, B subunit of Stx1.

Shiga toxin 1 (Stx1) is a virulence factor of enterohaemorrhagic Escherichia coli strains such as O157:H7 and Shigella dysenteriae. To prevent entry of Stx1 from the mucosal surface, an immunoglobulin A (IgA) specific for Stx1 would be useful. Due to the difficulty of producing IgA monoclonal antibodies (mAb) against the binding subunit of Stx1 (Stx1B) in mice, we took advantage of recombinant technology that combines the heavy chain variable region from Stx1B-specific IgG1 mAb and the Fc region from IgA. The resulting hybrid IgG/IgA was stably expressed in Chinese hamster ovary cells as a dimeric hybrid IgG/IgA. We separated the dimeric hybrid IgG/IgA from the monomeric one by size-exclusion chromatography. The dimer fraction, confirmed by immunoblot analyses, was used for toxin neutralization assays. The dimeric IgG/IgA was shown to neutralize Stx1 toxicity toward Vero cells by assaying their viability. To compare the relative effectiveness of the dimeric hybrid IgG/IgA and parental IgG1 mAb, Stx1-induced apoptosis was examined using 2 different cell lines, Ramos and Vero cells. The hybrid IgG/IgA inhibited apoptosis more efficiently than the parental IgG1 mAb in both cases. The results indicated that the use of high affinity binding sites as variable regions of IgA would increase the utility of IgA specific for virulence factors.

Introduction

Immunoglobulin A (IgA) is the major class of antibodies present on the mucosal surface as secretory IgA (SIgA). SIgA is believed to prevent pathogens and their virulence factors invading through the mucosal barrier.1,2

As a virulence factor of enterohaemorrhagic Escherichia coli (EHEC) strains such as O157:H7 and Shigella dysenteriae,3,4 we have been investigating Shiga toxin 1 (Stx1). Stx1 comprises one cytotoxic subunit and 5 binding subunits (Stx1B).5 We produced recombinant Stx1B as an antigen to investigate the immune response leading to efficient IgA production.6 The immunogenicity of Stx1B is not high enough to induce Stx1B-specific IgA responses efficiently in mice.7 This may indicate a limitation of vaccination against Stx1. On the other hand, preformed IgA against Stx1B may be useful for passive immunity in the form of a therapeutic antibody.

However, production of IgA monoclonal antibodies (mAb) turned out not to be straightforward, especially in the case of antigens with weak immunogenicity like Stx1B. In our case, we succeeded in the production of an Stx1B-specific IgA mAb, G2G7.8 However, this IgA mAb did not, but another IgG1 mAb, D11C6, did neutralize the toxicity of Stx1 holotoxin.9 To obtain antibodies with useful variable regions and with the IgA constant region, we produced a recombinant hybrid IgG/IgA, in which variable regions were from IgG1 mAb, while the heavy chain constant region was from IgA mAb.10 This hybrid IgG/IgA was shown to neutralize Stx1, of which the toxicity toward Vero cells was measured.11

Through expression of immunoglobulin heavy and light chains together with joining (J) chains in Chinese hamster ovary (CHO) cells, we were able to produce a dimeric form of the hybrid IgG/IgA. The dimerization of IgA is known to be required for the formation of SIgA.1,2 In CHO cells capable of stably
expressing the dimeric IgG/IgA, both dimeric and monomeric forms were present. After separation by means of size-exclusion chromatography, we demonstrated the dimeric form was 10-fold more effective than the monomeric one as to toxin neutralization. However, comparison of the dimeric IgG/IgA and parental IgG1 mAb in terms of toxin neutralization was not performed.

Stx1 is known to induce apoptosis of Burkitt’s lymphoma cells and kidney-derived Vero cells. In this study, we focused on comparison of the dimeric form of IgG/IgA and the parental IgG1 mAb as to toxin neutralization. We utilized 2 types of cells, Ramos cells (Burkitt’s lymphoma) and Vero cells, using 2 different assays that reflect apoptosis induction by Stx1 holotoxin.

**Results**

**Preparation of dimeric hybrid IgG/IgA by size-exclusion chromatography**

We previously established a CHO-K1 cell clone stably expressing dimeric hybrid IgG/IgA antibodies specific for Stx1B. This clone expresses heavy, light and J chains. Because the heavy chain consists of V_{H}, C_{L}, C_{A}2 and C_{A}3 domains, this antibody is able to dimerize through a J chain. A serum-free culture supernatant was prepared, concentrated and subjected to size-exclusion chromatography on Sephacryl S-300 (Fig. 1). Each fraction was examined by SDS-PAGE under non-reducing conditions, followed by immunoblotting with anti-IgA antibodies as a probe. The dimeric hybrid IgG/IgA (molecular mass higher than 220 kDa) was mainly separated in fractions 46 to 52. Some IgA molecules remain monomers in this clone. The monomeric hybrid IgG/IgA (molecular mass between 120 and 220 kDa) was found between fractions 52 to 57. To keep the incorporation of monomers as low as possible, we pooled fractions 47 to 50 in the present study. For biological assays, the antibody concentration in the pooled fraction was determined by sandwich ELISA.

**Preincubation with hybrid IgG/IgA dose-dependently neutralizes Stx1 toxicity toward Vero cells**

Vero cells are one of the cell lines sensitive to Stx1 toxicity. When Vero cells were cultured with 5 pg/ml of Stx1 holotoxin, cell viability decreased by 40%, as revealed by a cell viability assay (an MTT-like assay) that measures NAD(P)H-dependent cellular oxidoreductase activity by the reduction of water soluble tetrazorium salt (WST)-8. When Stx1 was treated with increasing concentrations of the dimeric fraction of hybrid IgG/IgA, the viability of Vero cells recovered (Fig. 2). Complete recovery was observed with more than 10 ng/ml of the hybrid IgG/IgA.

**Inhibition of Stx1-induced phosphatidylserine exposure on the Ramos cell surface by hybrid IgG/IgA**

Ramos cells are one of the Stx1-sensitive cell types from Burkitt’s lymphomas. Because of the nature of lymphoma cells, they are suitable for flow cytometry-based assays. Thus, cell surface exposure of phosphatidylserine was determined as an early event of apoptosis by flow cytometry. Apoptotic cells are recognized as cells that bind to annexin V but fail to incorporate...
propidium iodide (Fig. 3A). When Stx1 was pre-incubated with the dimeric hybrid IgG/IgA, and then the mixture of Stx1 (5 pg/ml) and the antibody (10 ng/ml) was added to the Ramos cell culture, apoptosis was inhibited by 98%. At the same concentration, IgG1 mAb (D11C6) inhibited apoptosis by 70%, whereas IgA mAb (G2G7) did not inhibit it at all. Dose-response studies revealed that the Stx1-induced apoptosis was more efficiently inhibited by the hybrid IgG/IgA than the parental IgG1 mAb (Fig. 3B). IgA mAb did not inhibit it up to 100 ng/ml.

**Inhibition of Stx1-induced caspase-3 activation in Vero cells by hybrid IgG/IgA**

Vero cells were also used to examine the effect of antibody treatment on the Stx1-induced apoptosis. Incubation of Vero cells with 5 pg/ml Stx1 resulted in caspase-3 activation. This was shown by the luciferase reaction on a substrate that was made available through cleavage of the caspase recognition sequence on the substrate precursor. Stx1 caused caspase-3 activation to a similar level to that with 20 μM camptothecin. When Stx1 was treated with increasing concentrations of the hybrid IgG/IgA, caspase-3 activation was dose-dependently inhibited (Fig. 4). At concentrations higher than 10 ng/ml of the hybrid IgG/IgA, complete inhibition was observed. When IgG1 mAb D11C6 was examined in parallel, caspase-3 inhibition was observed but 100 ng/ml was required for complete inhibition.

**Discussion**

Our previous studies demonstrated that the dimeric hybrid-IgG/IgA is more than 10-times more effective than the monomeric one as to toxin neutralization. The dimeric hybrid IgG/IgA was prepared by size-exclusion chromatography from a culture supernatant of CHO cells that express H, L and J chains. Because the monomeric hybrid IgG/IgA preparation was derived from CHO cells expressing only H and L chains, this preparation could not contain the dimeric form. That is, fair comparison was possible in terms of the antibody valence at the same constant region of IgA. However, it remains unknown whether or not the toxin neutralization activity was influenced by the difference in the Fc regions. Thus, we compared the parental IgG1 mAb and the hybrid IgG/IgA, which share the same variable region.

The results of our previous toxin neutralization assay involving Vero cell metabolic activity showed that more than 10 ng/ml of the dimorphic form of hybrid IgG/IgA induced complete inhibition of the toxicity of Stx1. As to the present dimer preparation, similar dose-dependent inhibition by the hybrid IgG/IgA was observed; that is 10 ng/ml of the hybrid IgG/IgA gave rise to complete neutralization in the MTT-like assay (Fig. 2).

To evaluate the neutralization activity of the hybrid IgG/IgA by assays other than the MTT-like assay, we examined the effect on the apoptosis induction by Stx1 holotoxin. We used 2 different assays involving different cell lines. For Ramos cells, phosphatidylserine exposure was detected as an early event of apoptosis. For Vero cells, caspase-3 activation was examined as a process involved in the apoptotic pathway. In both assays, 10 ng/ml of the hybrid IgG/IgA was shown to be sufficient to inhibit apoptosis induction by Stx1 completely. This is consistent with the results of the MTT-like assay. In contrast, this concentration is not sufficient for the parental IgG1 in either the assay involving Ramos or that involving Vero cells.

In the Ramos cell assay, no inhibition of apoptosis by IgA mAb was observed up to 100 ng/ml. The lack of toxin neutralization by IgA mAb is consistent with our previous findings. The lack of inhibitory activity of IgA mAb is not due to the absence of the dimeric form. The efficient inhibition by the hybrid IgG/IgA indicated that weak ability of toxin neutralization is not intrinsic to the IgA class. If appropriate variable regions are linked to IgA constant regions, efficient toxin neutralization can be attained.

To determine the relative efficacy of antibodies, caspase-3 activation in Vero cells was measured with a more sensitive assay. Camptothecin was used as a positive control showing the activation of caspase-3. Stx1 induced caspase-3 activation to a similar
level to that with camptothecin. In this system, the hybrid IgG/IgA completely inhibited caspase-3 activation at more than 10 ng/ml, whereas 100 ng/ml is needed for the parental IgG1 mAb to obtain the same level of inhibition. Thus, the dimeric hybrid IgG/IgA was 10-fold more efficient than the parental IgG1 mAb as to inhibition of caspase-3 activation in Vero cells. This is in parallel with our previous finding that the dimeric hybrid IgG/IgA was at least 10-fold more efficient than the monomeric form as to recovery of the Vero cell viability decrease induced by Stx1. The increased valence of antibodies seems to contribute to the lattice formation with antigens.

In conclusion, the dimeric form of hybrid IgG/IgA specific for Stx1B inhibited Stx1-induced apoptosis in 2 different cell lines, Ramos and Vero cells. The dimeric form of hybrid IgG/IgA inhibited apoptosis more effectively than the parental IgG1 mAb did. The results suggested that the utilization of high affinity binding sites as the variable regions for IgA might contribute to expansion of the IgA repertoire. This includes ones that are difficult to obtain by active immunization in vivo. The potential application of the hybrid IgG/IgA is to the neutralization of toxins on the mucosal surface of the intestine through oral administration. It should be noted that an appropriate choice of the variable regions would expand the range of application of the hybrid IgG/IgA to virulence factors of various pathogens.

Materials and Methods

Reagents
Shiga toxin 1 (Stx1) and the purified recombinant B subunit of Stx1 (Stx1B) were prepared as described previously.6,9 Mouse mAbs specific for Stx1B of IgA class (G2G7) and of IgG1 subclass (D11C6) were prepared as described previously.8,9 The expression of dimeric hybrid IgG/IgA specific for Stx1B in CHO-K1 cells was performed as described previously.10,11 A culture supernatant containing hybrid IgG/IgA was prepared in CD CHO-A medium (Life Technologies). Mouse myeloma proteins TEPC 15 (IgA, \(\kappa\)) and MOPC 21 (IgG1, \(\kappa\)), bovine serum albumin (BSA; Fraction V), and camptothecin were purchased from Sigma; purified goat anti-\(k\) chain and horseradish peroxidase (HRP)-goat anti-mouse IgA (\(\alpha\) chain-specific) from Southern Biotech; and purified rabbit anti-mouse IgG (\(\gamma\) chain-specific), HRP-goat anti-mouse IgG (\(\gamma\) chain-specific) and Medium 199 (M199) were purchased from Life Technologies; RPMI 1640 and kanamycin sulfate from Wako Pure Chemicals (Osaka, Japan); fetal bovine serum (FBS) from Hyclone; and N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) and a Cell Counting Kit-8 from DOJONDO.
(Kumamoto, Japan). The Cell Counting Kit-8 utilizes WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium) as a substrate. Sephacryl S-300 High Resolution was purchased from GE Healthcare; and Vivaspin 20–30 K and Vivaspin 500–30 K from Sartorius. Caspase-Glo® 3/7 Assay was purchased from Promega; and an FITC-Annexin V Apoptosis Detection Kit from BD PharMingen.

**Cells**

African green monkey kidney-derived Vero cells (American Type Culture Collection, ATCC) were cultured in M199 supplemented with 10 mM HEPES (pH 7.2), 10% FBS and 60 μg/ml kanamycin (10% FBS-M199). Burkitt’s lymphoma Ramos cells (ATCC) were cultured in RPMI 1640 supplemented with 10 mM HEPES (pH 7.2), 10% FBS and 60 μg/ml kanamycin (10% FBS-RPMI 1640). Cell cultures were performed at 37 °C under a humidified atmosphere of 5% CO2/95% air.

**Separation of dimeric hybrid IgG/IgA**

A serum-free culture supernatant of CHO-K1 cells producing dimeric hybrid IgG/IgA was concentrated by means of Vivaspin 20–30 K and Vivaspin 500–30 K, and the buffer was changed to 160 ml of phosphate-buffered saline containing 0.02% NaN3 (PBS-NaN3). The concentrated supernatant was separated on a column of Sephacryl S-300 (1.6 cm × 60 cm) equilibrated with PBS-NaN3. Fractions (1 ml each) were collected and OD280 nm was measured. To detect hybrid IgG/IgA in each fraction, 9 μl aliquots were subjected to SDS-PAGE (7.5% non-reducing Laemmli’s conditions; Mini-PROTEAN TXG Precast Gel, BIO-RAD) with MagicMark™ XP Western Protein Standards (Life Technologies) as molecular weight standards. After blotting onto a PVDF membrane (Millipore), the hybrid IgG/IgA was detected with HRP-goat anti-mouse IgA (1:1,000) using a chemiluminescence reagent (West Pico; Thermo Scientific Pierce) with a luminescence image analyzer, LAS-3000 (FUJI Photo Film, Tokyo, Japan). Fractions containing dimeric hybrid IgG/IgA were pooled and concentrated, and the buffer was changed to sterile PBS by means of a Vivaspin to remove NaN3, followed by sterilization by membrane filtration.

**ELISA**

To quantify assembled hybrid IgG/IgA and IgA mAb, a sandwich ELISA was employed. Thus, immobilized goat anti-mouse κ chain (1:1,000) was used as a capture antibody and HRP-goat anti-mouse IgA (1:1,000) for detection. BSA was used for blocking of nonspecific binding sites on the wells of an ELISA plate (Costar 9018, Corning). IgA myeloma TEPC 15 was used as a standard. For IgG1 mAbs, immobilized rabbit anti-mouse IgG (1:1,000) was used as a capture antibody and HRP-goat anti-mouse IgG (1:1,000) for detection. IgG1 myeloma MOPC 21 was used as a standard.

**Cell viability detection by MTT-like assay**

Vero cells were plated at 2 × 10⁴ cells/100 μl of 10% FBS-M199 into the wells of a 96-well plate (Falcon® 353072, Corning), and then cultured for 16 h. Stx1 (5 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 a 100-μl aliquot of the mixture of Stx1 and an antibody, the Vero cells were further cultured for 42 h. After the medium had been removed, 100 μl of M199 and 10 μl of Cell Counting Kit-8 (containing WST-8) were added to each well, and the cell culture was continued for 4 h. Cell viability was colorimetrically measured as described. Absorbance readings were made with a microplate reader (SUNRISE Rainbow RC-R; Tecan, Salzburg, Austria) at 450 nm with reference at 650 nm. Viability was expressed as the percentage of the control level (without toxin exposure).

**Annexin V binding assay**

Stx1 (1 pg) and varying amounts of an antibody (0.02 to 20 ng) were mixed and incubated for 1 h at 37 °C in 40 μl of 10% FBS-RPMI 1640. The mixture of
Stx1 and an antibody was added to 160 μl of a Ramos cell suspension (1.25 × 10^6 cells/ml) in 10% FBS-RPMI 1640. Thus, the final concentrations were 1 × 10^6 cells/ml, 5 pg/ml of Stx1, and 0.1 to 100 ng/ml of antibodies, respectively. The cells were cultured for 5 h at 37 °C, and then washed twice in PBS by centrifugation (200 × g for 5 min). The cells were then incubated with FITC-annexin V and propidium iodide for 15 min according to the manufacturer’s instructions. The cells were analyzed for the binding of annexin V and the incorporation of propidium iodide with a flow cytometer (FACS Canto II; BD Biosciences, San Jose, CA, USA).

**Caspase-3 activation**

Vero cells were plated at 1 × 10^4 cells/100 μl of 10% FBS-M199 into the wells of an opaque white 96-well plate (Falcon®, San Jose, CA, USA), and then cultured for 16 h. Stx1 (5 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 50-μl aliquot of the mixture of Stx1 and an antibody, the Vero cells were further cultured for 24 h. The Caspase-Glo reagent (50 μl) was added to each well according to the manufacturer’s instructions for the detection of activated caspase-3. Cells were incubated for 1 h at 37°C, and then signals were measured with a luminometer (Wallac 1420 ARVOsX; PerkinElmer Inc., Waltham, MA, USA). As a positive control, Vero cells were incubated with 20 μM camptothecin.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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