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Polymeric IgA Is Superior to Monomeric IgA and IgG Carrying the Same Variable Domain in Preventing Clostridium difficile Toxin A Damaging of T84 Monolayers

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The two exotoxins A and B produced by Clostridium difficile are responsible for antibiotic-associated colitis and antibiotic-associated colitis and is thought to account for 25% of antibiotic-associated diarrhea (1). Toxigenic strains of C. difficile produce two exotoxins that are involved in the pathogenesis of this diarrhea. Toxin A (308 kDa), an enterotoxin that causes fluid secretion and hemorrhage in animal models, is generally accepted as the primary toxin responsible for producing clinical symptoms (2). The second toxin, toxin B (279 kDa), is a cytotoxin detected by its cytopathic effects in cell culture, with little enterotoxicity in animals (3, 4). Both toxins cause mucosal damage and electrophysiological changes when applied to human colonic mucosal strips mounted in Ussing chambers (5). In this system, toxin B was found to be 10-fold more potent than toxin A in producing a transepithelial resistance drop. Using cultured nonpolarized Don cells, the ratio was as high as 1000-fold in favor of toxin B (6). In contrast, apical exposure of polarized human intestinal T84 cells showed that toxin A was 10-fold more potent than toxin B in inducing permeability defects (7, 8); this 10-fold greater sensitivity to toxin A is also observed in the animal intestine (9).

Binding of the toxins to cell surface exposed receptors constitutes the first step for enterotoxicity (10, 11). There have been a number of studies attempting to identify the cell receptors specific for each toxin (12–14). The trisaccharide Gal[^3]Gal[^β]4GlcNac[^β] appears to be the minimum receptor structure for binding toxin A (15), whereas no receptor for toxin B has yet been identified. The substance P receptor neurokinin-1 is required for toxin A-mediated acute inflammatory responses in mouse intestine (16). After binding to their putative receptors, toxins A and B enter the cell (17) and cause alterations to the actin microfilament network through glycosylation of the Rho protein family (18–20). Inactivation of Rho proteins alters epithelial permeability by loosening the tight junctions without producing cell death. The differing glycosyltransferase activity of toxins A and B appears to be the main determinant contributing to the difference in cytotoxic potency (6, 21).

Therapies other than antibiotics have been used in refractory or recurrent C. difficile infections. Anion-exchange resins binding to the toxins or competition by orally administered lactobacilli or nonpathogenic yeasts prove to be promising in the clinic (22–24). A number of clinical studies indicate that anti-toxin Ab responses in both serum and intestinal secretions may be protective, whereas an inadequate response predisposes to recurrent infection (25). Active immunizations using the C-terminal portion of toxin A (26, 27) and passive transfer of Abs to toxins A and B protect animals and prevent relapse of infection (28–30), yet a better molecular understanding of the molecular and cellular partners involved will help to design novel approaches for treatment and prevention. Epitopes have been mapped on the two toxins (31), but neutralizing epitopes have only been found associated with toxin A (32, 33), whereas IgG Ab binding toxin B epitopes was unable to inhibit its cytotoxicity in cell culture (34).

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In this paper, we have examined the capacity of three molecular forms (IgG, IgA<sub>mon</sub>, and IgA<sub>poly</sub>)<sup>a</sup> of a mAb specific for toxin A to neutralize the toxins applied either alone or together on the luminal (apical) or sera/osal (basolateral) side of cultured human intestinal T84 epithelial monolayers. The toxin A-specific Ab PCG-4 (IgG2a) was able to neutralize the combined action of the two toxins in the apical compartment only. This suggests that opening of the tight junctions by toxin A is a prerequisite for toxin B action. The neutralization properties of PCG-4 IgG2a were lost at 10 h of apical exposure and 6 h when the Ab was added basolaterally. Conversion of PCG-4 IgG2a into IgA<sub>mon</sub> with the same Fv domains extended protection for at least 24 h in both the apical and basolateral compartments. By comparing different Ig concentrations and kinetics of neutralization, we demonstrated that toxin A-specific IgA<sub>poly</sub> was at least four times more potent than IgG and IgA<sub>mon</sub>. The neutralizing capacity of IgA<sub>mon</sub> resembled that of IgG2a, thus showing that the avidity of IgA<sub>poly</sub> contributes to the increased biological activity of the Ab. In addition to better defining the mode of action of toxin B, the data provide solid molecular evidence that an efficacious vaccine should favor production of IgA<sub>poly</sub> on both sides of the mucosal epithelium.

Materials and Methods

Cells and proteins

The PCG-4 hybridoma cells (35) were provided by OraVax (Cambridge, MA). Human colon carcinoma T84 cells (CCL 248) and Chinese hamster ovary (CHO) dhfr<sup>−</sup> cells (CRL 9096) were purchased from American Type Culture Collection (Manassas, VA). Purified toxins A and B from the C. difficile strain VPI 10663 was provided by OraVax. Purified toxins were diluted in PBS to a final concentration of 0.51 mg/ml for toxin A and 0.31 mg/ml for toxin B and stored at −70°C. Production and purification of toxin A-specific PCG-4 IgG2a and recombinant chimeric IgA<sub>mon</sub> and IgA<sub>poly</sub> are described below.

Cell culture conditions

T84 cells were cultured and passaged in a 1:1 mixture of DMEM (1 g/l glucose) and Ham’s F12 medium supplemented with 10% FCS (Oxoid AG, Basel, Switzerland), 10 mM HEPES (pH 7.0), 1 mM sodium pyruvate, 2 mM glutamine, and 100 µg/ml each of penicillin/streptomycin at 37°C in a water-saturated atmosphere of 95% air and 5% CO<sub>2</sub>. To permit cell proliferation and establishment of tight monolayers, cells were seeded onto 12-mm polycarbonate membrane filters (0.4 µm pore Transwell; Corning) or Snapwell; Corning, at a density of 1 × 10<sup>5</sup> cells/filter. The culture medium was replaced freshly every 2–3 days as described (36). PCG-4 hybridoma cells were grown in 2-liter roller bottles in RPMI 1640 medium supplemented with 10% FCS (Myocyte; Life-Technologies, Zurich, Switzerland), 25 µM folic acid, 2 mM glutamine, 1 mM sodium pyruvate, and 100 µg/ml each of streptomycin-penicillin. CHO cells were cultured in α-MEM supplemented with 10% FCS (Flied, Logan, UT), 10 mM HEPES (pH 7.0), and 50 µg/ml gentamicin at 37°C in a water-saturated atmosphere of 95% air and 5% CO<sub>2</sub>.

Transepithelial resistance measurements

Transepithelial resistance (TER) was measured using a resistance system (Millicell-ERS; Millipore, Bedford, MA) for electrophysiological readings of filter cups. Cells with a number of passages ranging from 12 to 21 were grown for 12–20 days on Transwell or Snapwell membrane filters led to the establishment of TER values ranging from 1250 to 5000 Ω·cm<sup>2</sup>. Such resistance variation is attributable to monolayer to monolayer variation in tight junction permeability (37).

Purification of the PCG-4 Ab

The IgG2a Ab was purified from the hybridoma supernatant by protein A-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) following the manufacturer’s instructions. The eluate in 0.1 M glycine (pH 2.75) was immediately brought to pH 7.0 using 1.0 M Tris-HCl (pH 8.0). The elution buffer was exchanged for PBS by three subsequent washes in a Centriflo ultrafiltration (cut off, 50 kDa; Amicon, Beverly, MA), and the protein concentration was measured using the bicinchoninic acid assay (Pierce, Rockford, IL). Aliquots containing 25.3 ± 2.2 mg/ml of purified Ab were stored at 4°C. The Ag-binding activity of the purified Ab was assessed by ELISA using 100 ng of toxin A as coating Ag, 2.53 µg/ml purified Ab, biotinylated sheep anti-mouse Ig (Amersham Pharmacia Biotech) diluted 1:500, and streptavidin-HRP (Amersham Pharmacia Biotech) diluted 1:1000. Under these assay conditions, purified PCG-4 IgG2a yielded 1.998 ± 0.002 OD unit at 492 nm.

Generation of toxin A-specific recombinant IgA Abs

Rearranged genomic variable regions coding for the light (European Molecular Biology Laboratory access number, MMIGVKPCG, 835 bp) and heavy (European Molecular Biology Laboratory access number, MMIGVHPCG, 563 bp) chains from hybridoma PCG-4 were isolated (38) and cloned into expression vectors containing the genes for either the human α2m (1) or κ constant regions (39). IgA<sub>mon</sub> and IgA<sub>poly</sub> were produced in CHO cells cotransfected with expression vectors coding for chimeric mouse-human light and heavy chains, and the human J chain (pcDNAHygro-Jchain) as described (39). Following G-418 and hygromycin B selection for 2 wk, a clone (B2) was isolated and cultured in 175-cm<sup>2</sup> T-flasks to permit production of recombinant IgA. Crude supernatants were sterilized by filtration through 0.22-µm Millipore filters (Millipore), and stored at −20°C in the form of 45-ml aliquots. Quantitation of murine-human chimeric Abs in crude supernatant was performed using ELISA (39). The specific Ag-binding activity of the chimeric Abs was assessed using 100 ng of toxin A as coating Ag, 0.27 µg/ml of Ab, biotinylated rabbit anti-human α-chain IgG (Dako, Copenhagen, Denmark) diluted 1:1000, and streptavidin-HRP (Amersham Pharmacia Biotech) diluted 1:1000. Under these assay conditions, the chimeric IgA yielded 1.865 ± 0.009 OD unit at 492 nm.

Purification of toxin A-specific chimeric polymeric IgA Abs

Crude supernatants (5 × 45 ml) were concentrated 22.5× times using an Amicon Ultrafilter unit PM 10 (Amicon) and purified from serum proteins by chromatography on a Sephacryl S-300 column (1 × 2.6 cm; Amersham Pharmacia Biotech) equilibrated and run in PBS-0.02% sodium azide. Column fractions were analyzed by immunoblot of SDS/polyacrylamide gels under nonreducing conditions, and those containing separated IgA<sub>mon</sub> and IgA<sub>poly</sub> were pooled. IgA<sub>mon</sub> was concentrated by ammonium sulfate precipitation (final concentration, 33 g/l), resuspended in 1 ml of PBS, and desalted by filtration over a prepacked 2.5-ml PD 10 column (Amersham Pharmacia Biotech) equilibrated and run in PBS. Finally, the IgA solutions were sterilized by 0.22-µm filtration, aliquoted in siliconized 1.5-ml plastic tubes, and stored at 4°C.

Exposure of T84 monolayers to toxins and Abs

To evaluate the effect of toxin A or B on TER of T84 cell monolayers (results shown in Fig. 1), different concentrations of toxin A (range, 0.32–3.2 × 10<sup>−10</sup> M) or toxin B (range, 3.7–37 × 10<sup>−14</sup> M) were added to the upper or lower compartments of Transwell and incubated for 2.5 h. Dilutions of the toxins were conducted in complete cell culture medium. TER values were measured as indicated above using three independent replicate filters per experiment.

To examine whether toxin A can potentiate the action of toxin B after absoporation (results shown in Fig. 3), T84-coated filters were first preincubated for 1 h with 0.64 × 10<sup>−10</sup> M of toxin A before apical addition of 37 × 10<sup>−10</sup> M of toxin B. TER values were measured after 2.5 h and using five independent replicate filters per experiment. Control experiments included apical and basolateral addition of toxins A or B alone. The effect of blocking toxin A with 3.75 × 10<sup>−10</sup> M PCG-4 IgG2a before combination with toxin B was examined 5 h after addition to either the apical or basolateral compartments of the Ab-toxin mixtures.

To assess Ab-mediated protection as a function of time and degree of polymerization (results shown in Figs. 4 and 6), a constant concentration of toxin A (3.2 × 10<sup>−10</sup> M) was preincubated with different concentrations of Ab at room temperature for 30 min in a final volume of 30 µl. For the neutralization experiment shown in Fig. 4, PCG-4 IgG2a and recombinant IgA<sub>mon</sub> were used at a concentration of 7.5 and 1.5 × 10<sup>−10</sup> M. For the data presented in Fig. 6, IgG2a, IgA<sub>mon</sub>, and IgA<sub>poly</sub> Ab concentrations ranged from 0.23 × 10<sup>−10</sup> to 7.5 × 10<sup>−10</sup> M. Following apical or basolateral addition to the filters, TER values were measured at time 0 and after various

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*a Abbreviations used in this paper: IgA<sub>mon</sub>, monomeric IgA; IgA<sub>poly</sub>, polymeric IgA; S-Ab, secretory IgA; CHO, Chinese hamster ovary; Fv, variable domain of Ig; TER, transepithelial electrical resistance.*

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periods of incubation using three independent replicate filters per experiment. Control experiments were conducted in the absence of toxin or in the presence of the Ab preparations only.

Morphology

The cell monolayers cultured on filters were fixed for 1 h at 4°C with 3% paraformaldehyde in PBS. One half of each filter was embedded in methacrylate and 1-mm-thick sections were prepared and stained with toluidine blue. The other half was permeabilized with 2% Triton X-100 (Pierce) in PBS for 4 min, and cells were then incubated with rabbit anti-ZO-1 (40) (Zymed, South San Francisco, CA) in a humidified atmosphere. After washing, cells or sections were incubated with 10 ng/ml of fluorescein-conjugated anti-rabbit IgG. Preparations were examined under a Axioskop microscope (Zeiss, Seltbach, Switzerland) equipped for epifluorescence or by confocal laser scanning microscopy (Zeiss).

Results

Toxins A and B differentially affect the transepithelial resistance, tight junction organization, and integrity of T84 cell monolayers

In contrast to colonic cells in Ussing chambers, T84 cells allow continuous observation for up to 48 h and are sensitive to lower toxin doses (5). Thus, we selected this human cell line to conduct the series of experiments reported here. Because the effect of the two C. difficile toxins on T84 cell monolayers grown on permeable filters has been examined upon apical delivery exclusively, we first determined the polarity of their activity in the same set of experiments. In cultured T84 cell monolayers, previous results indicate that TER changes in responses to toxin A are a more sensitive indicator of barrier function than mannitol flux (8). We therefore measured the TER of the monolayer after continuous exposure for 2.5 h of increasing concentrations of toxin A (0.32–3.2 × 10^{-10} M) or B (3.7 × 10^{-10}–37 × 10^{-10} M) to the apical or basolateral compartment (Fig. 1). TER was already affected with the lowest concentration of toxin A used, and substantially reduced when the T84 monolayer was exposed to 1.9 × 10^{-10} M of toxin A. In controls, TER values remained above 1000 Ω.cm². In contrast, toxin B was without effect from the apical side, whereas when basolaterally applied, a marked drop in TER was observed with the lowest concentration tested. This first series of data suggest a so far unreported polarized mode of action for toxin B.

A drop in TER values is indicative of a loss of epithelial integrity. Thus, we analyzed the morphology of T84 monolayers exposed for 6 or 24 h to 3.2 × 10^{-10} M of toxin A added to the upper chamber of Transwell filters using an Axioskop device. At 6 h, toxin A caused a slight enlargement of the space between the epithelial cells without significant cell alterations (compare A and B of Fig. 2), whereas at 24 h, the monolayer was completely disorganized as reflected by swollen and vacuolated cells (Fig. 2C). Toxin A or B added to the basolateral side triggered changes similar to those of toxin A added apically, with a more rapid and pronounced effect for basolateral toxin B (data not shown). We next examined the organization of the tight junction network using a ZO-1-specific Ab and confocal microscopy. Tight junctions form a continuous network that seals the apex of the cells (Fig. 2D). After 6 h of apical treatment with toxin A, the tight junction network was minimally disrupted (Fig. 2E), whereas exposure for 24 h caused a complete disorganization with disappearance of ZO-1 labeling (Fig. 2F). When applied basolaterally, toxin A completely disrupted the tight junction network after 6 h (Fig. 2G), and there was no further change with prolonged treatment (Fig. 2H).

In conclusion, our data extend previous studies concerning the polarity of T84 epithelial susceptibility to the two toxins (7, 8). Using cells grown in parallel and the same toxin preparation, we found that the effect of toxin B on the tight junctions is only seen when the toxin is added basolaterally. At this point, these results suggest that toxin B needs a damaged epithelium to exert its cytotoxic activity.

Toxin A makes toxin B delivered in the apical surface cytotoxic

Consistent with this hypothesis, it is conceivable that the opening of the tight junctions by toxin A will allow toxin B to reach the basolateral membrane where its cytotoxic effect will take place and potentiate the action of toxin A on the damaged monolayer. The concentration of toxin A (0.64 × 10^{-10} M) was adjusted so that the reduction of TER was only partial and thus permitted to detect subtle changes due to the addition of 37 × 10^{-10} M toxin B in the apical compartment. In the presence of the two toxins, there was a more pronounced reduction of TER after 2.5 h of incubation compared with toxin A alone (Fig. 3A, lanes A/~ and A + B/~). The more marked combinatory effect of both toxins was still significant after 5 h (p > 0.05), yet toxin A begins to exhibit a cytotoxic effect by itself. In agreement with data in Fig. 1, toxin B alone did not have any effect on the TER (Fig. 3A, compare lane B/~ with lane /~). Conversely, the addition of toxin A, toxin B, or a combination of the two toxins to the basolateral compartment (Fig. 3B, lanes A/~, B/~, and A + B/~) induced a drastic drop in TER values. Therefore, our finding that apical coapplication of toxin B and toxin A synergistically affect the T84 monolayer TER confirms the possibility that toxin B exerts its action to the basolateral surface after the initial disruption of tight junctions by toxin A.
We thus postulated that an Ab able to neutralize toxin A should protect the epithelial cell monolayers when the two toxins are added together apically. We tested the protection afforded by PCG-4 IgG2a Ab, which recognizes two epitopes within the repeating units of toxin A (35) and is known to neutralize the toxin’s enterotoxic activity in rabbit ileal loops (41). As shown in Fig. 3A (lanes A+/B+), PCG-4 mAb added with toxins A and B to the apical compartment protected the monolayers to the same extent as when mixed with toxin A alone (Fig. 3A, lane A+/). This argues in favor of the necessary role of toxin A in making apical toxin B active against the epithelial cell monolayer. Consistently, PCG-4 IgG2a was inefficient in preventing the cytotoxic effect of toxin B when the latter was added basolaterally to the T84 monolayers and incubated for either 2.5 h (black bars) or 5 h (gray bars). Mean values of three independent experiments are shown. Preincubation with the IgG2a Ab before addition to the cells was performed in lines marked with a +. The shaded area indicates the limit of confidence of the TER values of three individual control filters incubated for the same period of time in the absence of toxin.

**FIGURE 2.** The morphological effects of toxin A on T84 monolayers grown on Transwell filters. A. Monolayers of T84 cells were left untreated or incubated with 3.2 × 10^{-10} M of toxin A added apically for either 6 h (B) or 24 h (C). Filters were recovered and 0.5-μm sections were stained with toluidine blue. D–H, Confocal microscopy of fixed and permeabilized T84 monolayers labeled with an Ab directed against the tight junction-associated protein ZO-1. D, Untreated cells. E and F, Cells exposed apically to 3.2 × 10^{-10} M of toxin A for 6 and 24 h, respectively. G–H, Cells incubated basolaterally with 3.2 × 10^{-10} M of toxin A for 6 and 24 h, respectively. The lower panels represent a section conducted along the XY plan. The thin white line represents the site where XZ sections shown in the upper part were performed.

**FIGURE 3.** Effects of toxins A and B alone or in combination and of PCG-4 Abs on T84 monolayer transepithelial resistance. Toxin A (0.64 × 10^{-10} M), toxin B (37 × 10^{-10} M), or a mixture of both toxins were added apically (A) or basolaterally (B) to Transwell chambers containing T84 monolayers and incubated for either 2.5 h (black bars) or 5 h (gray bars). Mean values of three independent experiments are shown. Preincubation with the IgG2a Ab before addition to the cells was performed in lines marked with a +. The shaded area indicates the limit of confidence of the TER values of three individual control filters incubated for the same period of time in the absence of toxin.

**Time dependence of toxin A neutralization by IgG2a Abs**

T84 cell monolayers were treated with toxin A [3.2 × 10^{-10} M; the highest toxin A concentration tested in the dose-response curve (Fig. 1)] and purified IgG2a (7.5 or 15 × 10^{-10} M) for 3, 4, 6, 8, 10, and 24 h. On the apical side, protection was efficient for the first 6 h at both Ab concentrations, whereas a drop in TER below the reference value was observed at 10 and 24 h (Fig. 4A). Consistently, little alteration in the tight junction network was observed after 6 h; at 24 h, the morphological consequence were much more pronounced (Fig. 4, B and C). In contrast, protection on the basolateral side was efficient for the first 4 h only (Fig. 4D), as reflected by the severe disruption observed at 6 h and disappearance of the ZO-1 signal at 24 h (Fig. 4, E and F). A differential apical and basolateral expression of toxin receptor(s) might explain variations in kinetics of cellular damages. In the absence of a clearly identified receptor, we postulated that the limitation in the duration of protection might well result from a dynamic exchange of toxin A between the Ab and its binding sites on the epithelial cells. We addressed this possibility by comparing the protection capacity of IgG2a Abs expressing the same Ag-binding specificity.

**Toxin A-specific IgA Abs enhance efficacy and duration of neutralization**

To examine the neutralizing capacity of monomeric and polymeric Abs, we cloned the genes coding for the variable regions of the
PCG-4 Ab into vectors carrying human constant heavy and light chain genes (39). The constructs were expressed together with a human J chain expression vector in stably transfected CHO cells. IgAd/p and IgA m Ab molecules were recovered from clone B2 and separated by sieving chromatography (Fig. 5 A). The identity of the Ab molecular forms in column fractions corresponding to the peaks was confirmed by Western blot analysis of samples subjected to SDS-PAGE under nonreducing conditions (Fig. 5 B).

Potpurification identical amounts of IgA m and IgA d/p were checked by ELISA for their toxin A-binding activity (H. Stubbe, unpublished observations). To specifically assess the role of each Ig as such, we did not combine IgA with secretory component, as the latter has been shown to bind to toxin A (42).

When toxin A (3.2 × 10⁻¹⁰ M) was added with IgA d/p (7.5 or 15 × 10⁻¹⁰ M) to either the apical or basolateral side of T84 cell monolayers, we found that IgA d/p fully neutralized toxin A efficiently in both compartments at the two molar ratios tested. Protection at the apical surface was extended from 8 to at least 24 h. In the basolateral compartment, the neutralizing effect of the Ab was prolonged from 4 to at least 24 h (Fig. 4, G and J). Maintenance of TER values correlated with the integrity of the tight junction network (Fig. 4, H and I and K and L). Direct comparison of the ZO-1 pattern at 6 and 24 h following apical or basolateral application of the Ab-toxin A complex indicated that, at Ab:Ag molar ratios of either 2.3 or 4.6, IgA d/p fully protected the epithelial monolayer. In agreement with our working hypothesis, this suggests that the Ab-toxin A complex is much more stable when cross-linking is mediated by the multivalent IgA d/p Ab.

Since IgG and IgA d/p carry the same Fv fragments and therefore have the same basal affinity for the Ag, we next tested whether the difference in avidity accounts for the enhanced duration of protection. Toxin A (3.2 × 10⁻¹⁰ M) was added with decreasing concentrations of IgG, IgA m, and IgA d/p in the apical compartment and TER was monitored after 2.5, 4, and 6 h (Fig. 6). At 2.5 h, IgA d/p concentrations as low as 0.46 × 10⁻¹⁰ M neutralized the toxin, whereas either monomeric Ig at the same low concentration gave TER values below the range of confidence. A similar capacity to maintain TER values was obtained with 7.5 × 10⁻¹⁰ M of either monomeric Ig. This indicates that in this experimental setting, IgA d/p is 16-fold more efficacious than its monomeric counterpart, and that 1 mol of IgA d/p can neutralize 6 mol of toxin A (see Discussion for possible explanations). At 4 h, 1.9 × 10⁻¹⁰ M of IgA d/p still fully prevented toxin A-induced TER drop. This shows that IgA d/p remains superior to monomeric Ig in neutralizing toxin A after longer exposure time. At 6 h, a slight molar excess of IgA d/p (3.7 × 10⁻¹⁰ M) over toxin A was still able to maintain TER to control values, whereas 7.5 × 10⁻¹⁰ M monomeric IgG2a or IgA m could no longer neutralize toxin in the assay. This suggests that free, non-neutralized toxin A capable to exert its destructive effect becomes available to the T84 cell surface more slowly when the Ag is complexed to IgA d/p. In addition, direct comparison between different isotypes with the same variable domains indicate that IgA d/p provide more efficacious and prolonged protection on both surfaces of the epithelial monolayer than monomeric Ig. The biologic implication of these findings is discussed below.
Discussion

The aim of this study was to compare at the molecular level the neutralizing capacity of IgG and IgA using a well-documented model of infection, i.e., the effect of *C. difficile* toxin A and B on polarized epithelial T84 cells. While establishing the system in the laboratory, we could reproduce the behavior of both toxin on T84 cells under similar conditions (7, 8). In addition, we found that toxin B applied basolaterally for 5 h is much more potent in destroying epithelial cell monolayers than the same concentration delivered apically (Figs. 1 and 2). One implication is that the two toxins, despite structural similarities, recognize distinct epithelial cell receptors with the expression of the receptor for toxin B restricted to the basolateral cell surface. An inaccessibility (or lack) of receptors for toxin B on enterocytes has been suggested to explain the discrepancy in intestinal activities of toxins A and B (43). Identification of this putative receptor will require that polarized cells are used to permit its initial biochemical characterization. The difference in sensitivity of apical and basolateral surfaces to pathogens has already been reported (44) and suggests that cell polarity of intact epithelia is likely to contribute to mucosal defense. Furthermore, by using short times of incubation (2.5 and 5 h) and suboptimal concentrations of toxin A, we have shown that toxin B is rapidly active when delivered at the apical surface (Fig. 3). This would agree with the strongly potentiating effect of toxin B on sublethal amounts of toxin A eliciting enterotoxic effects in rabbits, rats, mice, and hamsters (4, 9). Thus, the culture model mimics very well the in vivo situation seen in experimental animals. Moreover, the experiments performed with anti-toxin A IgG2a imply that neutralization of toxin A might be sufficient to prevent initial damage and basolateral delivery of toxin B to its preferential site of action. Because toxin B has been shown to induce the release of proinflammatory cytokines and chemokines by lamina propria leukocytes, including mast cells (45), macrophages (46, 47), and neutrophils (48, 49), inhibition of its diffusion into the serosal compartment should reduce tissue damage and propagation of the bacterium.

Another issue challenged by our results is the type of optimal detection which should be conducted on stool specimens subjected

![FIGURE 5. Preparation of Ig_Ap and Ig_Am Abs carrying the Fv from PCG-4. A, Molecular sieving chromatography of the different molecular forms of IgA molecules expressed by CHO cells. Arrows correspond to the peak elution and numbers to the molecular mass (kDa) of globular proteins used to calibrate the column. B, A selection of column fractions was run under nonreducing conditions on a 6% polyacrylamide gel and transferred onto blotting membrane. The identity of the proteins in the first three peaks was confirmed by immunodetection using antiserum to the α-chain.](http://www.jimmunol.org/)

![FIGURE 6. Comparative analysis of the neutralizing capacity of IgG2a, Ig_Am, and Ig_Ap Abs. Toxin A (3.2 × 10^-10 M) along with decreasing amounts of purified Abs were added apically to T84 cell monolayers. TER values were measured at time 0 ( ), after 2.5 h ( ), 4 h ( ), and 6 h ( ).](http://www.jimmunol.org/)
to clinical analysis. Many hospital laboratories do not perform assays for both toxins and consequently it is important to determine which *C. difficile* toxin(s) is(are) prevalent and responsible for pathogenesis (50). In pediatric patients, testing for toxin A would have detected 50% of the infection, whereas testing for toxin B alone would have detected *C. difficile* in 82% of the cases (51). Given that toxin A at low concentration makes epithelial surfaces sensitive to toxin B, our data suggest that a negative test for toxin A cannot exclude *C. difficile* infection. Failure to diagnose *C. difficile* in the early stage of infection may result in worsening of the patient condition and increased costs for subsequent treatment.

Direct comparative studies of local immunity directed against infections of mucosal surfaces have been hindered in the past because of the problem of obtaining sufficient amounts of Ab molecules with the same specificity, but of distinct isotypes. The advantages of recombinant Abs are numerous: their degree of purity and achievable concentration are well above those of Ab purified from body fluids including bile, colostrum, nasal, or intestinal washings; purified Ig samples are devoid of cellular/glandular contaminants such as other Igs and lymphokines known to modulate epithelial function (52); the specificity and isotyping of the Ab produced in CHO cells is identical to that found in natural secretions (39). Clearly, the recombinant Ig molecules produced in this study represent the best tool so far to strictly investigate how IgG, IgAm, and IgAdp carrying the same Fv fragments neutralize a bacterial toxin on the surface of reconstituted monolayers of epithelial T84 cells.

Our data provide strong molecular evidence that the avidity of IgAdp Ab increases both its intrinsic efficacy as predicted, but also the duration of its neutralizing capacity. Although not expected from in vitro experiments, this observation suggests a mechanism whereby the favored tethering of toxin A on IgAdp limits the productive contact between the toxin and the epithelial cells leading to G protein activation and subsequent disruption of the epithelial monolayer. Consistent with this mechanism derived from the pure in vitro experimental setting used here, colonic aspirates from healthy individuals frequently contain secretory IgA (S-IgA) Ab molecules to toxin A able to block the binding of toxin A to its intestinal receptor (53). Moreover, colostal S-IgA has been shown to neutralize both toxins A and B (54), which may explain the low frequency of detection of *C. difficile* toxins in newborns and young infants (51). Interestingly, in the absence of mucus and peristaltic movement, IgAdp Ab remains much more efficient than monomeric IgG, indicating that immune exclusion believed to prevent diffusion of cross-linked Ags is also operative in vitro.

Remarkably, IgAdp was still operative at 24 h, whereas the IgG molecule was no longer able to prevent the toxin A effects. Thus, neutralization by IgAdp and IgG are quantitatively different phenomena. The nature of this difference was examined by preparing IgAm, which behavior was found to be close to that of the IgG counterpart. Therefore, the polymeric nature of IgAdp, along with its concomitant increased avidity, explains the difference in biological function. One can argue that the receptor on epithelial cells can 1) act as scavenger for the Ab molecules, and thus preclude their neutralizing activity, or 2) favor cellular entrance of Ab-Ag complexes, thus potentiating the action of the toxin. First, we were unable to detect FcRn (55) on the surface of T84 cells (B. Courthésy, unpublished observations), implying that the concentration of IgG Ab remains stable and that IgG-Ag complexes cannot exert any intracellular activity. Second, the very weak expression of pIgR at the basolateral surface of T84 does not reduce the amount of IgAdp capable of neutralizing toxin A, nor does it transcytose Ab-Ag complexes to a detectable level. Finally, the expression of the Fcα receptor (CD89) is limited to cells of the myeloid lineage (56) and does not intervene in the process under study here.

The data in Fig. 5 indicate that dimeric IgA-containing fractions also contain a significant amount of higher molecular weight IgA polymers. This can explain why we observed that 1 mol of recombinant IgAdp could neutralize 6 mol of toxin A (Fig. 4), the valence of the Ig molecules in the assay being on average higher than 4. Carbohydrate moieties on IgA could also contribute nonspecific binding sites for toxin A (57), and thus enhance toxin neutralization as already reported for *Escherichia coli* type 1 fimbriae lectin Ag (58). In intestinal secretions, IgAm molecules are associated with the secretory component, which is heavily glycosylated (59). Given that the secretory component is a potent binder of toxin A (42), this may help to tether even more toxin A on the Ab molecule. In addition to blocking bacterial attachment (60), this might represent another mechanism whereby sugars on Ig molecules can exert their biological function.

Based on our observations, it appears that an efficient *C. difficile* vaccine should elicit Abs that neutralize toxin A in the gut lumen and inactivate both toxins A and B in the lamina propria. Neutralization of pathogens or toxins in the gut lumen is best accomplished by S-IgA, which are produced as IgAdp and transported in secretions by receptor-mediated transcytosis (61). Local IgA production and protective secretory immune responses in mucosal tissues require mucosal vaccination (62, 63), yet this concept appears to be challenged in the case of *C. difficile* vaccination. Indeed, Formalin-inactivated culture filtrates from a highly toxigenic *C. difficile* strain induced high S-IgA responses, but low protection, when administered rectally and intragastrically, whereas intranasal, i.p., and s.c. routes were 100% protective against death but only partially protective against diarrhea (64). Although a combination of nasal and peritoneal immunization fully protected hamsters from both death and diarrhea (64), the same group also reported that rectal immunization in conjunction with i.m. vaccination provided full protection; protection was associated with high levels of toxin-neutralizing Abs in serum (65). Rabbits inoculated orally with an attenuated *Vibrio cholerae* strain expressing a truncated form of toxin A induced both a systemic and mucosal protective immunity against this toxin (27). Together, these experiments indicate that optimal protection against *C. difficile* infection requires Abs in both the luminal and serosal compartments, consistent with our in vitro findings. Significantly, in patients with diarrhea, IgAm and IgAdp in the serum, not IgG, have been implicated in *C. difficile* neutralization (66). This is consistent with the observation that IgA-associated immunity to mucosal Ags can complement that of S-IgA in the defense against pathogens (67).

In conclusion, we demonstrate that an Ab specific for *C. difficile* toxin A protects human colonic epithelial T84 cell monolayers against both toxins A and B when the Ab acts in the luminal but not in the serosal compartment. We provide evidence that increasing the avidity of the Ab enhances both efficacy and duration of protection. Although suggested in previous studies (68), our data represent the first demonstration that the neutralizing capacity of model Ab molecules with the same Fv fragments is dependent on their isotype and degree of polymerization. The good parallel observed in vivo data and the results reported here makes it possible to draw this conclusion, and further suggests that a potent vaccine against *C. difficile* should elicit a strong mucosal, polymeric IgA-mediated immune response.
References


68. Taylor, H. P., and N. J. Dimmock. 1985. Mechanism of neutralization of influenza virus by secretory IgA is different from that of monomeric IgA or IgG. J. Exp. Med. 161:198.