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Selective Neutralization of a Bacterial Enterotoxin by Serum Immunoglobulin A in Response to Mucosal Disease

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One-third of convalescent-phase serum samples (6 of 18) from patients with *Clostridium difficile*-associated diarrhea demonstrated neutralization of the clostridial enterotoxin, toxin A. Although appreciable amounts of toxin A-specific immunoglobulin G (IgG) and IgA were present in these sera, the ability to neutralize the cytotoxic activity of toxin A on OTF9-63 cells in vitro was confined to the IgA fraction and the IgA1 subclass in serum samples from all six patients. In contrast to the patients with *C. difficile* diarrhea, this activity was present in both the IgA and IgG fractions in sera from two *C. difficile*-infected patients without diarrhea, one of whom presented with a splenic abscess. Sera and purified IgA which neutralized the cytotoxicity of toxin A on OTF9-63 cell cultures in vitro also neutralized the enterotoxicity of toxin A in rabbit ileal loops in vivo. This activity was not Fc dependent, since IgA retained neutralizing activity after pepsin digestion and $F(ab)_2$ purification. The transition from nonneutralizing toxin A-specific IgA in the acute-phase sera to neutralizing specific IgA in the convalescent-phase sera was accompanied by a shift from a polymeric to a predominantly monomeric form of specific IgA. However, the neutralizing activity in convalescent-phase sera was present as both monomeric and polymeric IgA. Convalescent-phase sera from other patients with *C. difficile* diarrhea that failed to neutralize toxin A also failed to produce a predominantly monomeric-form specific IgA response. We conclude that serum IgA, not IgG, characteristically neutralizes toxin A in patients with *C. difficile* diarrhea who develop neutralizing systemic responses. This neutralization of an enteric bacterial toxin is a unique and selective role for serum IgA which provides a novel functional link between the systemic and mucosal immune systems.

Immunoglobulin A (IgA) responses in serum often accompany both systemic and mucosal infections (2, 12, 28, 32). However, production of serum IgA appears to be regulated independently from mucosal IgA production (4). Serum IgA differs from mucosal IgA in its molecular form (primarily monomeric versus polymeric), distribution of subclasses (90% IgA1 versus 35 to 70% IgA1), and cellular origins (marrow and lymph node versus mucosal-associated diffuse lamina propria cells, respectively) (16, 35, 38). Moreover, few selective functional activities have been ascribed to pathogen-specific IgA in serum, whereas several distinct roles have been attributed to mucosal IgA (9, 18, 31, 38, 43).

Mucosal IgA mediates its protective functions by inhibition of adherence by pathogens, neutralization of viruses and toxins, and interaction with nonspecific host immune factors (18, 38, 43). Polymeric IgA in mucosal tissues may remove foreign antigens from the lamina propria and neutralize intracellular viruses by utilizing the polymeric Ig receptor for transport across epithelial cells (15, 29). A similar method for antigen clearance may operate in the hepatobiliary system as has been demonstrated in mice (34). In contrast, serum IgA does not readily facilitate the major immune functions of complement activation and phagocytosis performed by serum IgG (18, 43). Serum IgA may abrogate or enhance the immune response to systemic infections (10, 13, 36), but a specific relationship between serum IgA and mucosal infections has not been well characterized.

We describe a novel functional role for serum IgA. Following infection with *Clostridium difficile*, a mucosal bacterial pathogen, serum antibodies neutralize the cytotoxic and enterotoxic properties of the major *C. difficile* virulence factor, toxin A. The ability of these serum antibodies to neutralize the toxin resides most consistently in the IgA, not the IgG, serum fraction. Moreover, the development of neutralizing IgA over time is accompanied by a shift in the predominant molecular form of toxin A-specific IgA from polymeric to monomeric. These findings establish a selective functional link between serum IgA responses and mucosal infections.

MATERIALS AND METHODS

Subjects and sera. Serum samples were tested from 25 patients with *C. difficile* diarrhea (7 acute-phase, 7 convalescent-phase, and 11 paired acute- and convalescent-phase specimens) from whom acute-phase sera were obtained within 1 week of positive stool culture and convalescent-phase sera were obtained 3 to 8 weeks later (14). Patients with *C. difficile* diarrhea had six or more unformed bowel movements in 36 h, both stool culture and stool cytotoxin assay positive for *C. difficile*, and no other explanation for diarrhea. In addition, sera were obtained from 10 patients asymptomatically colonized (fecal excretors) with *C. difficile* in whom a toxigenic isolate was recovered from two consecutive stool cultures (14) and one patient who presented without diarrhea but with a splenic abscess from which *C. difficile* was recovered (39). Written informed consent was obtained from the subjects, and the study was approved by the Human Subjects Subcommittee at the VA Medical Center. Sera were stored at −20°C until testing.

ELISA for toxin A-specific Ig. Levels of toxin A-specific Ig were detected by a solid-phase enzyme-linked immunosorbent assay (ELISA) with 96-well microtiter plates (Immulon II; Dynatech Laboratories, Chantilly, Va.) coated with toxin A (1.5 μg/ml in carbonate buffer) as described previously (14). Toxin A (purified

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by D. M. Lyerly, Virginia Polytechnic Institute, Blacksburg) was homogeneous by crossed immunoelectrophoresis and produced a single band by analytical polyacrylamide gel electrophoresis (PAGE). Sera or purified Ig fractions (as described below) were added to duplicate wells in serial dilutions and incubated at room temperature for 2 h. Ig reactive with toxin A was detected with horseradish peroxidase-conjugated affinity-purified goat anti-human IgA or IgG (BioSource, Burlingame, Calif.). The assay is specific for toxin A as demonstrated by competitive inhibition with toxin A and heterologous antigens (14). Toxin A-specific IgA1 and IgA2 antibodies were detected as described above, with mouse monoclonal anti-human IgA1 or anti-human IgA2 (1:100; clones B3506B4 and A9604D2, respectively; Southern Biotechnology Associates, Birmingham, Ala.) and horseradish peroxidase-conjugated goat anti-mouse IgG1 (Southern Biotechnology). Specific antibody concentrations were determined by interpolation from absorbance values of control IgA1 and IgA2 standards that defined the linear part of a semilog dilution curve. The IgA subclass standards were assayed in wells coated with affinity-purified anti-human IgA (BioSource; 1:2,000). The sensitivity of both assays was 5 ng/ml. The specificity of the subclass assay was confirmed by use of monoclonal IgA1 (generous gift of A. Dalmasso, University of Minnesota, Minneapolis) and IgA2 (Biodesign International, Kennebunkport, Maine) standards in both assays. No cross-reactivity was detected between the IgA1 and IgA2 subclass ELISAs at IgA1 and IgA2 concentrations ranging from 0.001 to 1.0 μg/ml (data not shown).

Neutralization of toxin A. In vitro, neutralization of toxin A cytotoxic activity was determined with OTF9-63 cells derived from a mouse teratocarcinoma line that expresses a carbohydrate receptor for toxin A as described previously (14, 42). Sera or purified IgG or IgA was incubated at 37°C for 1 h with an equal volume of toxin A, the concentration of which was fivefold higher than the amount of toxin required to cause rounding of 90% of the cells. After incubation, the mixture was added to OTF9-63 cell culture, and the cells were examined for cytopathic changes after 18 h. All samples were coded, distributed randomly on the plate, and examined blindly. Neutralization was defined as <10% cell rounding after exposure to toxin and Ig specimen. A negative control for each sample (Ig alone) and positive controls (serial toxin A dilutions only) were included on each assay plate.

In vivo, neutralization of toxin A enterotoxic activity was determined by a rabbit ileal-loop assay described previously (14, 23). Briefly, toxin A (5 μg) was incubated with an equal volume of serum or purified IgG or IgA restored to the original serum concentrations for 1 h. After incubation, the mixture (1 ml) was injected into ligated rabbit ileal loops, and the enterotoxic response was measured after 12 h. Each loop was separated by intervening noninjected loops. Portions of the isolated ileal-loop segments were stored in formalin for histologic studies after the segment length and fluid volume were measured.

Antibody purification. IgG was affinity purified from 1 ml of patient serum with a protein G column (MAbTrap G; Pharmacia LKB Biotechnology, Piscataway, N.J.) washed with 20 mM sodium phosphate (pH 7.0). IgG was eluted with 0.1 M glycine HCl (pH 2.7), the fractions were neutralized with 1.0 M Tris (pH 9.0), and the IgG-containing fractions were combined and concentrated with a phosphate-buffered saline (PBS; pH 7.0) buffer exchange (Centriprep-30 concentrator; Amicon Corp., Beverly, Mass.)

IgA was affinity purified from IgG-depleted sera with agarose-bound jacalin (Pierce, Rockford, Ill.) as the ligand. Jacalin is a lectin with specificity for galactose residues on the hinge region of IgA1 (19). Pooled and concentrated void-volume fractions from the protein G column were applied to the jacalin column. IgA was eluted from the column with 0.1 M melibiose, and the elution fractions were combined and concentrated as described above. No attempt was made to purify IgA2 from sera because toxin A-specific IgA2 was detected infrequently and at low levels (see Table 1).

$F(ab)_2$ purification. Purified IgA (1.2 mg) was dialyzed against a 20 mM sodium acetate buffer (pH 4.5), incubated with 0.025 ml of a slurry of immobilized pepsin (9,000 U of pepsin activity per ml of slurry; Pierce) for 12 h at 37°C, and neutralized with 10 mM Tris-HCl (pH 7.5). The digested IgA was separated from the immobilized pepsin gel by centrifugation. The pepsin-digested IgA was fractionated on a column (1.5 by 85 cm) of Sephacryl S-200 (Pharmacia) equilibrated in PBS to remove residual Fc fragments. Elution fractions (2.5 ml) containing $F(ab')_2$ were combined and concentrated with a Centriprep-30 concentrator (Amicon). Analysis of the pepsin-digested IgA by sodium dodecyl sulfate (SDS)-PAGE under nonreducing conditions confirmed the degradation of the IgA to a homogeneous fragment with a molecular weight of ~110,000 (data not shown) (30).

IgA fractionation by molecular form. Intact affinity-purified IgA was fractionated by size by gel filtration chromatography (Sephacryl S-300 HR column [1.5 by 90 cm; Pharmacia] equilibrated with PBS), and the elution fractions (2.5 ml) were tested for toxin A-specific IgA by ELISA. The column was calibrated with polymeric IgA and monomeric IgA standards (generous gifts of J. Mestecky, University of Alabama, Birmingham). In some experiments, IgG-depleted sera were substituted for affinity-purified IgA. Analysis of these fractions by ELISA gave identical results to those obtained with purified IgA. For each of three samples, the polymeric and monomeric fractions of IgA were separated, concentrated, and tested for neutralizing activity in the OTF9-63 cell culture assay. Fractions between the polymeric and monomeric peaks which contained overlapping polymeric and monomeric IgA were not included in either separation. In addition, the separated polymeric and monomeric forms of IgA were analyzed by nonreducing SDS-PAGE (3% stacking–5% resolving gel) after incubation at 100°C for 2 min in a sample buffer containing 10 mM iodoacetamide, bromophenol blue, and glycerol. Five micrograms of each sample was loaded per gel lane. Following the electrophoresis at 50 V for 3 h, the gel was stained with Coomassie brilliant blue R-250.

To verify that the high-molecular-weight toxin A-specific IgA detected was polymeric IgA rather than IgA-immune complexes, we dissociated potential immune complexes prior to gel filtration. A serum specimen in which the toxin A-specific IgA was primarily polymeric was acidified by buffer exchange with 0.1 M sodium acetate (pH 4.1) (20, 37). The column was also equilibrated and recalibrated with the same buffer, and the elution fractions were neutralized with 1.0 M Tris-HCl (pH 9.0).

Statistical analysis. Toxin A-specific IgA1 levels were correlated with serum neutralization titers by regression analysis with the StatView 4.0 statistics program (Abacus Concepts, Inc., Berkeley, Calif.).

RESULTS

In vitro neutralization of *C. difficile* toxin A cytotoxicity by class-specific Ig from infected patients. Neutralizing activity was detected in 1 of 18 acute-phase serum samples and 6 of 18 convalescent-phase serum samples from 25 patients with *C. difficile* diarrhea, in 1 of 10 asymptomatic *C. difficile* excretors, and in 1 patient with a *C. difficile* splenic abscess. To determine which class of serum Ig was responsible for the neutralization of toxin A, we purified the IgG and IgA from these sera by affinity chromatography. Analysis of the elution fractions from the protein G and jacalin chromatographic separations by ELISA demonstrated efficient separation of the Ig classes and a 1,000-fold purification of IgG and IgA with respect to each other and to IgM (Fig. 1).

The neutralizing activity resided in the IgA fraction of all six convalescent-phase serum samples from the patients with *C. difficile* diarrhea who developed neutralizing responses. In contrast, none of the IgG fractions from these sera demonstrated neutralization (Fig. 2; Table 1). Consistent with these results, although only one of four acute-phase serum samples tested from these same patients neutralized toxin A, it was again the IgA, not the IgG, fraction which demonstrated neutralization.

Sera from two patients with other manifestations of *C. difficile* infection showed neutralizing activity by both IgA and IgG: one patient with a splenic abscess and the other with asymptomatic intestinal colonization (Table 1, subjects 7 and 8). Therefore, in sera with neutralizing activity, IgA was responsible for some or all of this activity in all eight serum samples, whereas IgG showed neutralizing activity in only two patients, both of whom had nondiarrheal *C. difficile* infections.

In vivo neutralization of toxin A enterotoxicity by class-specific Ig. The enterotoxic effect of toxin A in vivo may be more relevant to human illness than the cytotoxic effect of toxin A in vitro. Therefore, IgA which neutralized toxin A in vitro was also tested for neutralization of the effects of toxin A in vivo on isolated rabbit ileal loops. Twelve hours after inoculation with toxin A, the ileal segment was grossly dilated with viscous hemorrhagic fluid within the lumen (Fig. 3). Preincubation of toxin A with IgA purified from convalescent-phase serum from a patient with *C. difficile* diarrhea (Table 1, subject 1) showed obvious attenuation of the enterotoxic response. In contrast, preincubation of toxin A with IgG purified from that same serum or a protein control (bovine serum albumin [BSA]) showed overt hemorrhage and fluid accumulation.

Histologic changes paralleled the gross pathology and secretory response (Fig. 4). Ileal segments exposed to toxin A showed severe mucosal damage with hemorrhagic necrosis. Preincubation of toxin A with the purified serum IgG showed no abrogation of this damage. However, preincubation of toxin A with purified serum IgA showed only limited mucosal damage with acute inflammation (Fig. 4). These results show that IgA from patients convalescent from *C. difficile* diarrhea neu-

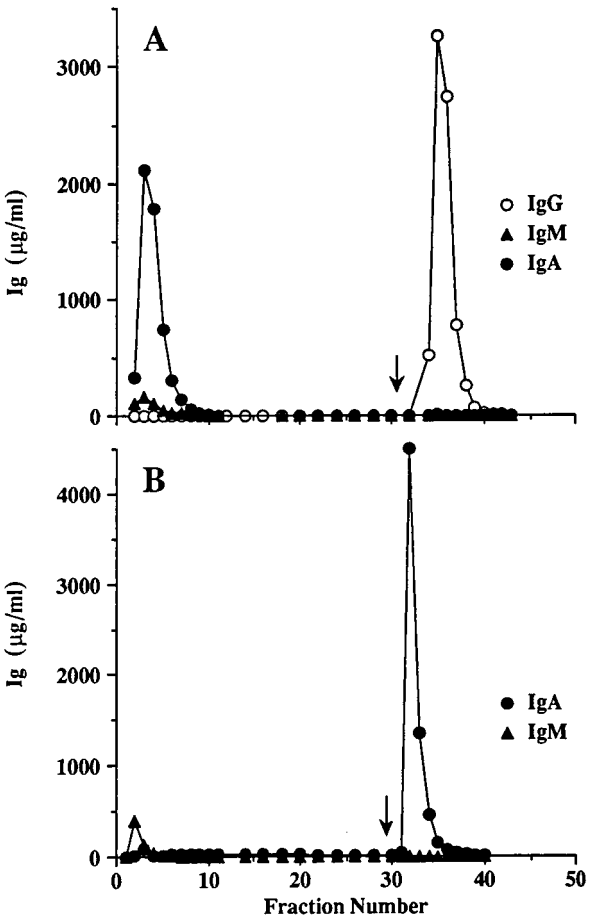

FIG. 1. Sequential purification of IgG and IgA from serum. Class-specific Ig levels in the elution fractions of serum following protein G affinity separation (A) and of IgG-depleted serum following jacalin affinity separation (B) are shown. An arrow marks initiation of the elution buffer for each column.

tralize much of the enterotoxic and secretory effects of toxin A in vivo as well as the cytopathic effects of toxin A in vitro.

Association of toxin A neutralization with the F(ab)$_2$ fragment of IgA. To determine whether the IgA neutralization of toxin A was dependent on an intact Fc component, purified IgA from a patient with a high serum neutralization titer (Table 1, subject 8) was digested with pepsin, and the Fcα fragments were removed by gel filtration. This IgA-derived F(ab′)$_2$ fragment retained reactivity with toxin A by ELISA as well as its in vitro cytotoxic neutralizing activity (data not shown). Therefore, the neutralization of toxin A by IgA represented a specific antibody-antigen interaction in this specimen and was not due to interaction with Fcα.

Association of toxin A neutralization with the IgA1 subclass. To characterize the subclass restriction of the IgA response to toxin A, sera were tested for toxin A-specific IgA1 and IgA2 by subclass ELISA. The majority of toxin A-specific IgA was restricted to the IgA1 subclass (Table 1). Specific IgA2 was detected at low levels in only 3 of the 11 serum samples tested (Table 1). Levels of specific IgA2 remained low in the IgG-depleted, non-jacalin-binding fractions of selected sera, and these fractions did not neutralize toxin A. Levels of toxin A-specific IgA1 correlated with serum neutralization titers when sera with neutralizing IgG were excluded ($r^2 = 0.610$; $P = 0.02$).

Association of toxin A neutralization with a shift in the predominant molecular IgA form. To study the association of the molecular form with the function of toxin A-specific IgA, monomeric and polymeric IgA fractions were separated by gel filtration from the sera of selected patients. The specific IgA was predominantly monomeric in all serum samples with neutralizing activity from the symptomatic patients (one acute-phase serum sample [subject 4] and four convalescent-phase serum samples [subjects 1, 3, 4, and 6] [Table 1]) as well as in the neutralizing serum from the asymptomatic *C. difficile* excretor (Table 1, subject 8). In contrast, the specific IgA was predominantly polymeric in two acute-phase serum samples without neutralizing activity from these same symptomatic patients (Table 1, subjects 1 and 3). Thus, in two patients with diarrhea, the change from nonneutralizing to neutralizing specific IgA in acute- and convalescent-phase sera, respectively, was associated with a shift from a predominantly polymeric to a monomeric form of specific IgA as well as an increase in the level of toxin A-specific IgA1 (Fig. 5 shows data from subject 3). Only those sera, both acute and convalescent phase, which showed a predominance of monomeric-form specific IgA demonstrated neutralizing activity. Conversely, each specimen with a predominance of polymeric-form specific IgA showed no neutralizing activity. These findings were confirmed with acute- and convalescent-phase serum samples from two patients with *C. difficile* diarrhea who did not develop neutralizing antibodies; specific IgA in each was predominantly polymeric (data not shown). Therefore, in each of 10 serum samples tested for neutralization and molecular form, the predominant molecular form of toxin A-specific IgA (monomeric or polymeric) predicted the neutralization results (neutralization or nonneutralization, respectively).

To exclude the possibility that the size of the nonneutralizing toxin A-specific Igs represented immune complex aggregates rather than polymeric IgA, the IgA was acidified prior to gel filtration. That the acidified IgA retained its large molecular size and did not dissociate into monomeric forms demonstrated that the nonneutralizing IgA was polymeric (data not shown).

We next determined which of these molecular forms of IgA was responsible for the neutralizing activity. We tested independently purified polymeric and monomeric IgA fractions from three serum samples for neutralizing activity. No overlap in size was detected in these separated fractions by nonreducing SDS-PAGE (data not shown). Both the separated polymeric and monomeric IgA fractions from two neutralizing convalescent-phase serum samples neutralized the toxin (Table 1, subjects 3 and 6; Fig. 5), whereas neither the monomeric nor polymeric fractions from a nonneutralizing acute-phase serum sample showed any activity (Table 1, subject 3; Fig. 5). Therefore, the development of toxin A neutralization was associated with a shift in the predominant molecular form of specific IgA, but the neutralizing activity was present in both the monomeric and polymeric forms of the molecule. Thus, the shifts in form and function of toxin A-specific IgA, from predominantly polymeric to monomeric and from nonneutralizing to neutralizing, are temporally but not structurally related.

DISCUSSION

This study demonstrates a novel functional role for serum IgA, neutralization of a bacterial toxin, that parallels a known function of mucosal secretory IgA (18, 43). The primary role of serum IgA has been presumed to be anti-inflammatory because of its relative lack of Fc-mediated functions (18, 36). We demonstrate that pathogen-specific serum IgA has a newly

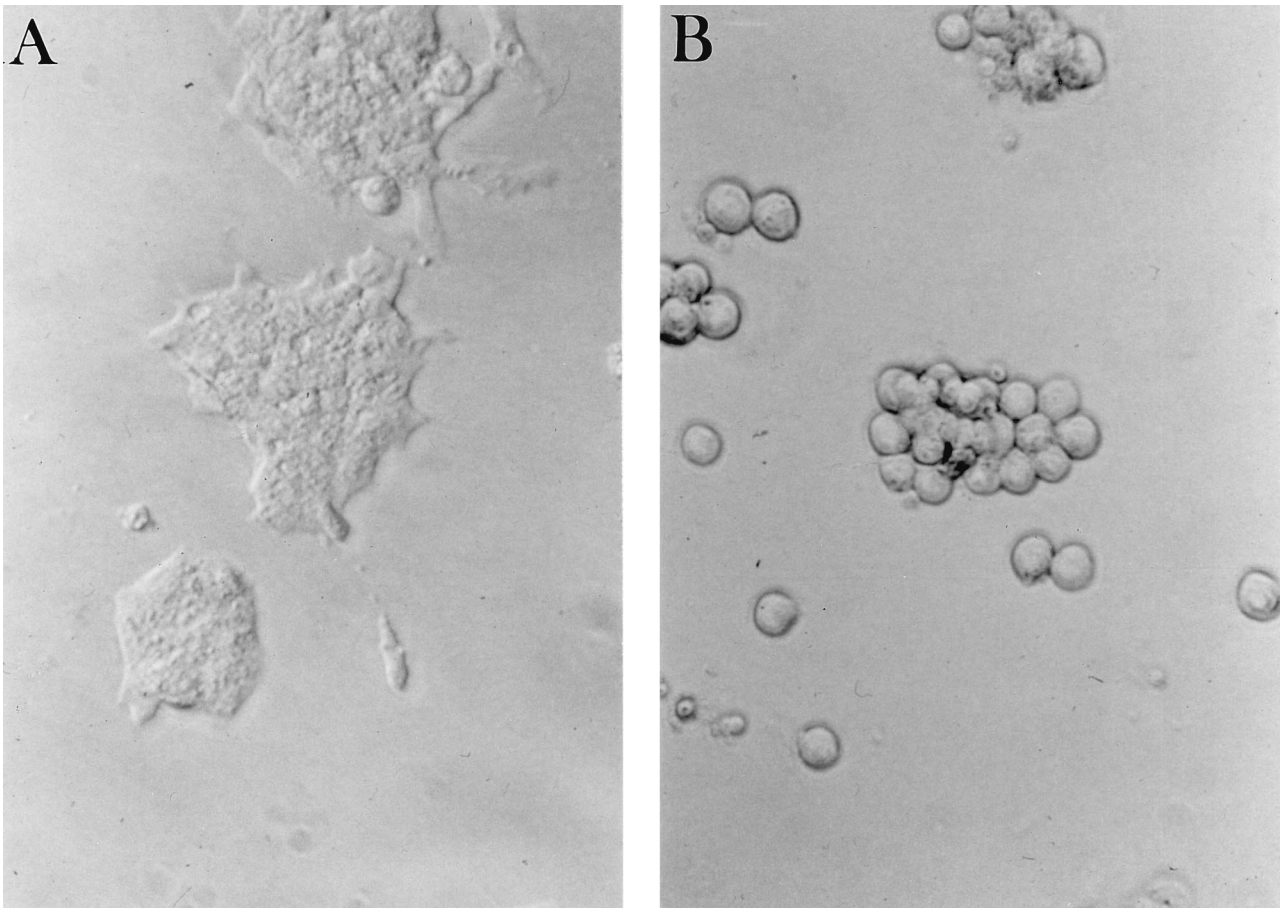

FIG. 2. Cytotoxic effects of toxin A on OTF9-63 cells. OTF9-63 teratocarcinoma cell clusters after exposure to toxin A preincubated with convalescent-phase serum IgA from a patient with *C. difficile* diarrhea (A) and after exposure to toxin A alone (B) for 18 h are visible. OTF9-63 cells normally grow in cell clusters (as opposed to confluent monolayers) with morphology identical to that shown in panel A.

defined, non-Fc-mediated function, i.e., toxin neutralization, which may limit systemic inflammation or damage (43). IgA in the sera of patients convalescent from *C. difficile* diarrhea neutralized both the cytotoxic and enterotoxic properties of toxin A, and this neutralizing function was retained after removal of Fc by pepsin. To our knowledge, this is the first demonstration of neutralization of a bacterial toxin by serum IgA.

We demonstrated previously that patients with *C. difficile* diarrhea developed systemic and mucosal antibody responses to toxin A (14). Convalescent-phase sera from one-third of the

TABLE 1. Toxin A neutralizing ability and levels of toxin A-specific IgA in sera from patients infected with *C. difficile*

Subject no.	Sample phase	Syndrome	Neutralization by serum (titer)	Neutralization by affinity purified Ig[a]		Toxin A-specific serum IgA level (μg/ml)	
				IgG	IgA	IgA1	IgA2
1	Acute	Diarrhea	0	0	0	0.14	0.00
	Convalescent		1:4	0	+	7.21	0.10
2	Acute	Diarrhea	0	0	0	0.68	0.00
	Convalescent		1:4	0	+	ND[b]	ND
3	Acute	Diarrhea	0	0	0	0.85	0.00
	Convalescent		1:8	0	+	3.14	0.00
4	Acute	Diarrhea	1:4	0		1.13	0.00
	Convalescent		1:4	0	+	2.04	0.00
5	Convalescent	Diarrhea	1:2	0	+	0.80	0.25
6	Convalescent	Diarrhea	1:32	0	+	8.73	0.00
7	Convalescent	Splenic abscess	1:64	+	+	11.30	0.35
8		Asymptomatic excretor	1:32	+	+	12.93	0.00

[a] Symbols: 0, no neutralization; +, neutralization.
[b] ND, not done.

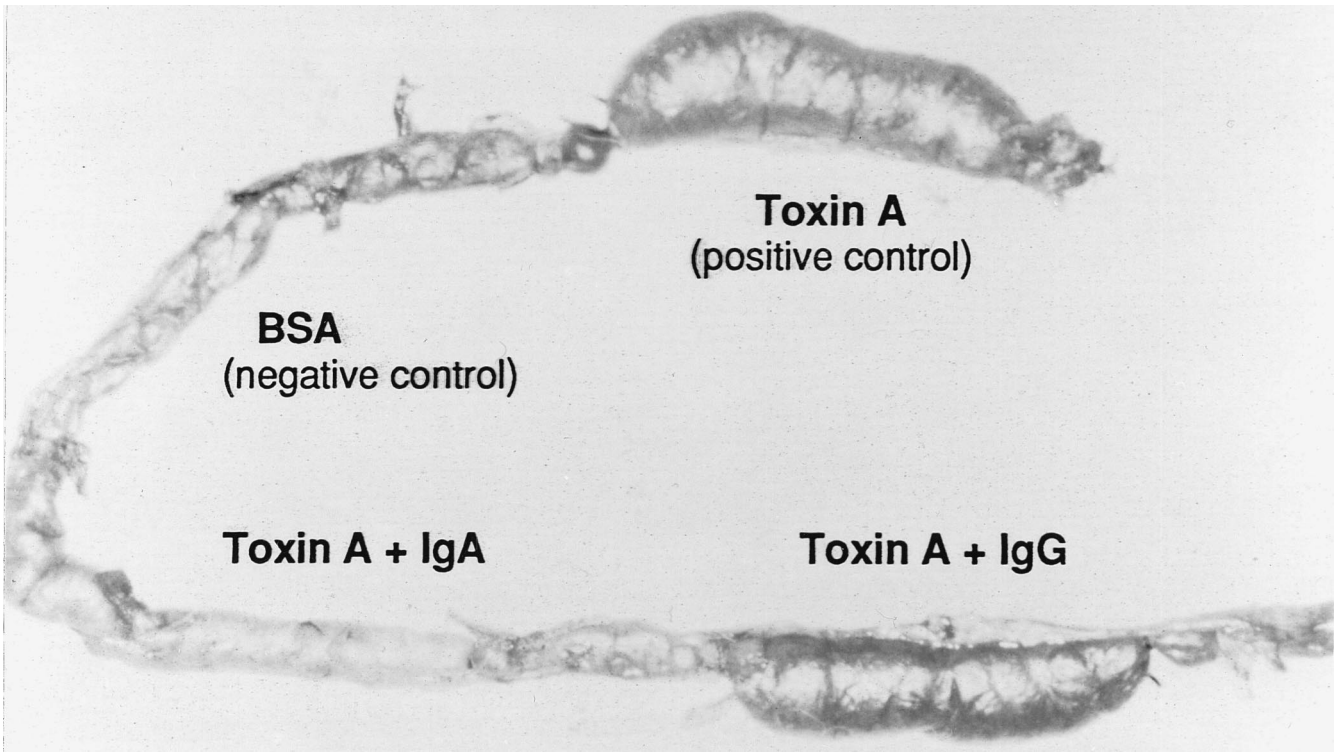

FIG. 3. Enterotoxic effect of toxin A on isolated rabbit ileal-loop segments. The ileal segments were dilated and grossly hemorrhagic 12 h after inoculation with either toxin A (5 μg/ml) preincubated with BSA (5 mg/ml) or toxin A preincubated with IgG affinity purified from the convalescent-phase serum from a patient with *C. difficile* diarrhea. In contrast, the ileal segment inoculated with toxin A preincubated with IgA affinity purified from the same patient serum was identical in gross appearance to a control segment inoculated with BSA alone. The gross enteropathic effects were confirmed by measurements of fluid accumulation in the lumen. The ratios of volume (milliliters) to length (centimeters) of the ileal segments were greater in the segments inoculated with toxin A plus BSA and toxin A plus IgG (0.5 and 0.4, respectively) than in the segments inoculated with toxin A plus IgA or BSA alone (0.01 and 0.00, respectively).

patients with *C. difficile* diarrhea neutralized toxin A, and the neutralization titers correlated with both the toxin A-specific IgG and IgA levels as measured by ELISA. Specific IgG levels also correlated with IgA levels, but as we now have demonstrated, only serum IgA, not IgG, was responsible for the neutralization in those patients with diarrhea. Although the specific mucosal secretory IgA levels showed the same temporal pattern as that of the serum antibody response, neutralization could not be demonstrated with these mucosal specimens which were obtained by oral colonic lavage. Improved mucosal specimen collection and processing techniques may be required to demonstrate whether secretory IgA neutralizes toxin A following natural infection. Additionally, the serum antibody response did not appear to alter the clinical course of the intestinal infection but, rather, reflected the severity of illness as measured by the presence or absence of diarrheal relapse. Nevertheless, serum antibody responses to mucosal infections may mediate other important functions such as immune clearance of toxins that reach the systemic circulation.

A unique aspect of our findings is the relatively selective neutralizing activity of systemic IgA, rather than IgG, in response to this typically noninvasive mucosal pathogen. Specific serum Igs, including IgA, have been detected in response to other enteric pathogens (2, 12, 28, 33). Neutralizing serum responses have been detected after natural infection with *Vibrio cholerae* and after enteral immunization with cholera toxin, but the Ig class has not been specified (11, 27, 33). However, these systemic responses to mucosal exposure are usually of low magnitude compared with the high level of neutralizing IgG elicited following parenteral immunization with cholera toxin (11). Neutralization by immune sera of the vacuolating cytotoxin produced by the gastric pathogen *Helicobacter pylori* is mediated predominantly by IgG (5). Whereas specific serum IgM has been shown to neutralize Shiga toxin in response to shigella diarrhea, an invasive enteritis (17), our patients showed no appreciable IgM response (14). The role of IgA in the serum neutralizing responses to other enteric pathogens, such as rotavirus, has not been characterized (3). Our data suggest the presence of a functional link between local mucosal infections and systemic IgA, rather than IgG, responses.

Cells producing serum IgA and those producing mucosal IgA are compartmentalized separately within the body (4). Although it might be hypothesized that specific IgA detected in serum in response to enteric infection is derived from luminal mucosal sources, a large body of evidence suggests that secretory IgA is not normally transferred (actively or passively) from the lumen into the systemic circulation (4, 7). Only trace amounts of secretory IgA are present in normal human sera, although some patients with liver disease or human immunodeficiency virus infection have increased secretory IgA levels (7, 44). In addition, the toxin A-neutralizing IgA from the patients with *C. difficile* diarrhea in this study was obtained approximately 1 month after the infection, when symptoms had resolved, presumably in association with resolution of mucosal inflammation and disruption. Our characterization of this toxin A-neutralizing IgA as primarily monomeric and of the IgA1 subclass is most consistent with the molecular forms of IgA in serum. However, the IgA1-restricted response to toxin A may also reflect the nature of the antigen rather than the source of

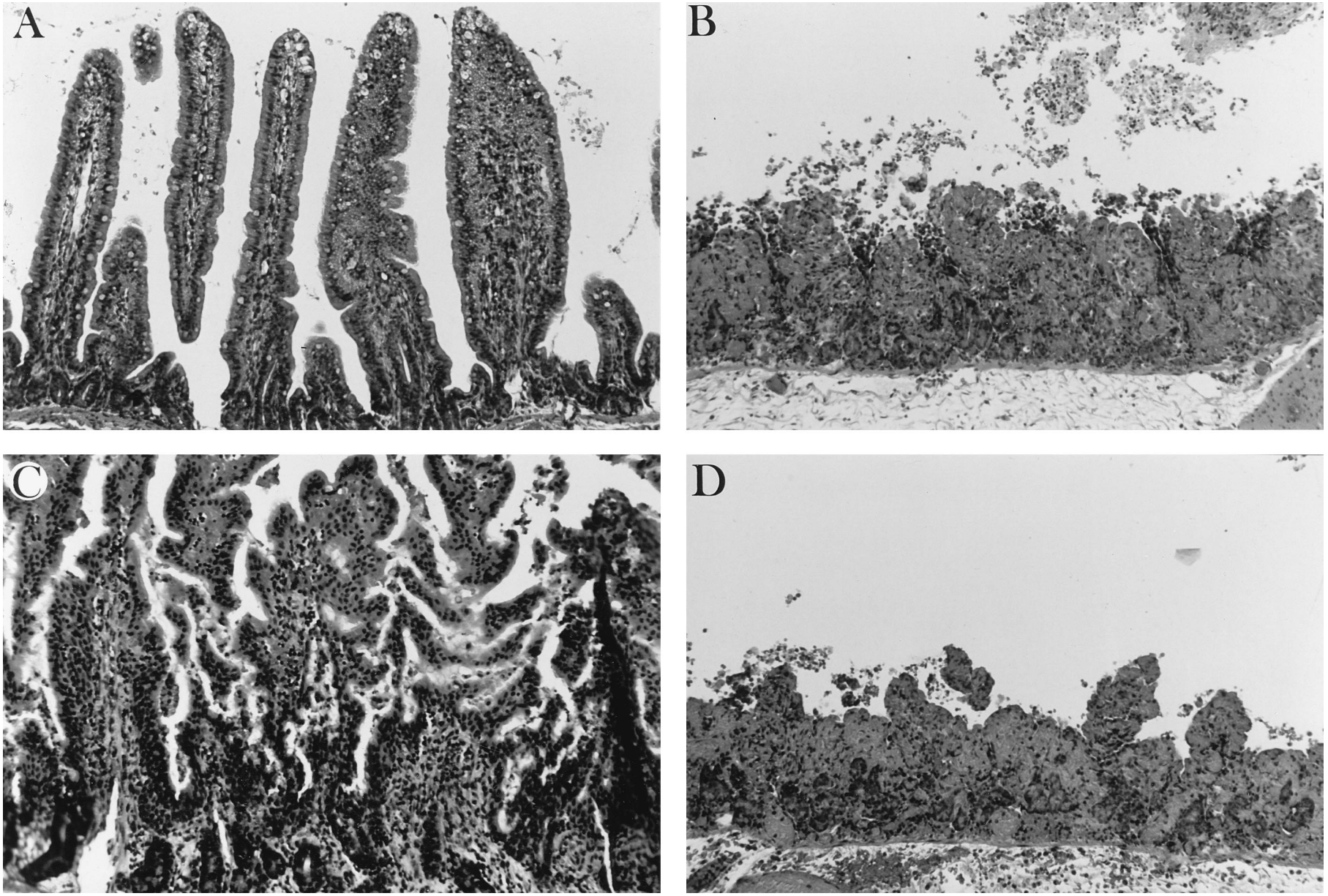

FIG. 4. Histologic effects of toxin A on isolated rabbit ileal-loop segments 12 h after inoculation. (A) Normal ileum in the negative control segment inoculated with BSA; (B) severe mucosal damage with hemorrhagic necrosis in the positive control segment inoculated with toxin A; (C) limited mucosal damage with acute inflammation in the segment inoculated with toxin A preincubated with IgA purified from the convalescent-phase serum from a patient with *C. difficile* diarrhea; (D) severe mucosal damage with hemorrhagic necrosis in the segment inoculated with toxin A preincubated with purified IgG from the same patient serum. Stain, hematoxylin and eosin; magnification, ×154.

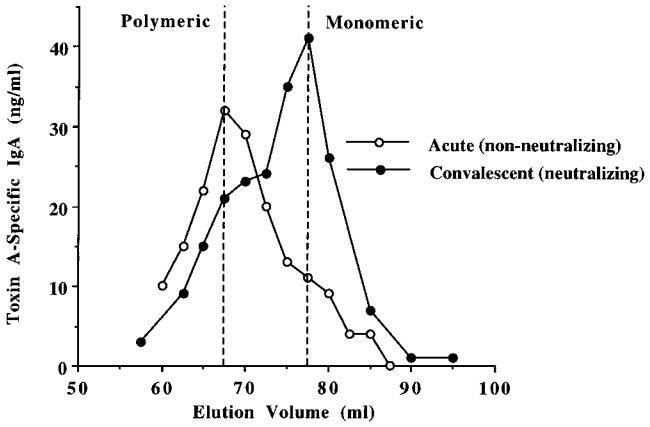

FIG. 5. Molecular form of toxin A-specific IgA in acute- and convalescent-phase sera from a patient with *C. difficile* diarrhea. Toxin A-specific IgA levels are shown in the elution fractions of these sera (Table 1, subject 3) fractionated by Sephacryl S-300 HR gel filtration. Dashed lines indicate the elution peaks of the polymeric and monomeric IgA standards. The toxin A-specific convalescent-phase serum IgA which neutralized toxin A was predominantly monomeric, whereas the toxin A-specific acute, nonneutralizing IgA was predominantly polymeric. The polymeric and monomeric fractions of each serum sample were also analyzed separately for neutralizing activity. Pooled monomeric and polymeric fractions from the convalescent-phase sera each neutralized toxin A, whereas neither of the pooled fractions from the acute-phase sera neutralized toxin A.

the antibody. Protein antigens preferentially induce IgA1, whereas polysaccharide antigens may elicit relatively greater proportions of specific IgA2 (35).

The source of toxin A-specific IgA in serum may have originated from cells within the intestinal mucosa, bone marrow, or in lymphoid tissues intermediate in location between the mucosa and systemic sites, such as mesenteric lymph nodes. The bone marrow is the primary source of serum IgA, of which 90% is monomeric. Although only polymeric IgA is transported into the intestinal lumen by secretory component, 40% of cultured mucosal lymphocytes also produce monomeric IgA (21). In addition, individual IgA-producing cells may be capable of secreting both monomeric and polymeric IgA, and therefore, the molecular form of IgA may not necessarily indicate the source of the Ig (41). It is also possible that some of the IgA-producing lymphocytes stimulated at the mucosal surface, the majority of which are destined to home back to the mucosa after systemic transit and maturation, may escape this route and migrate to the bone marrow and spleen (1).

Evidence of systemic exposure to toxin A in *C. difficile*-infected patients would lend support to a systemic source for toxin A-specific IgA. Unlike cholera toxin, toxin A is a unique enterotoxin which produces extensive mucosal damage in addition to fluid secretion and which may reach the systemic circulation (24). Despite the rarity of bacteremia and extraintestinal infections associated with *C. difficile* (22), systemic manifestations of fever and leukocytosis are found in 30 to 50% of patients with *C. difficile* diarrhea (8). Moreover, bacterial antigens may penetrate into mucosal tissues even in the absence of invasion by the whole bacteria (25). Therefore, these specific, functionally active serum IgA responses may result from systemic exposure to toxin A.

Although toxin A-specific IgA was primarily monomeric in the convalescent-phase neutralizing sera, the nonneutralizing responses in acute-phase sera from the patients with *C. difficile* diarrhea were primarily polymeric. Precise quantitation of polymeric IgA remains problematic, and the amount of polymeric IgA as measured by radioimmunoassay and other assays may be underestimated (6). The values for specific IgA concentrations of the gel filtration fractions were determined in our assay by interpolation from the values of a polyclonal IgA standard (predominantly monomeric) and may have underestimated the polymeric IgA concentrations. However, the shift in pattern of the polymeric to monomeric IgA response from the acute- to the convalescent-phase neutralizing sera was dramatic (Fig. 5; other data not shown). This shift has also been documented following other infections and antigenic challenges by immunization. Natural infections by viral or bacterial pathogens such as *Campylobacter jejuni* elicit transient polymeric IgA responses (28, 32). Immunization by either oral or parenteral routes also transiently stimulates polymeric serum IgA (26, 27, 40). We confirm this shift from toxin A-specific polymeric IgA in acute-phase sera to specific monomeric IgA in convalescent-phase sera, but we also observed that the shift was associated with an increase in levels of toxin A-specific IgA whereas others have observed this shift to be accompanied by a decrease in specific antibody levels with infections or immunization in humans (26–28). This difference emphasizes the transient nature of specific polymeric IgA early in the immune response to antigens in general and the rise in specific monomeric IgA in a subset of patients with *C. difficile* diarrhea.

We demonstrate that this shift in molecular form was accompanied by a transition from nonneutralizing to toxin A-neutralizing activity and that neutralization correlated with levels of toxin A-specific IgA1. However, the neutralizing activity was not limited to monomeric IgA but rather was present in both molecular forms of IgA. Neutralization of toxin A by both forms of IgA in the convalescent-phase sera is consistent with the demonstration in one patient that neutralization was not Fc dependent and, therefore, was likely due to specific antibody-antigen interaction. Thus, the structural and functional changes are related temporally to the maturation of the IgA response to *C. difficile* toxin A, but the two events may not be interdependent and the shift in molecular form may be less significant than changes in the variable region of IgA.

In summary, sera from a subset of patients with a mucosal enteric infection by *C. difficile* neutralize the effects of the major toxin produced during that infection, toxin A. The toxin A-neutralizing activity in sera from patients with *C. difficile* diarrhea was restricted typically to the IgA class. Only a patient with systemic infection (a *C. difficile* splenic abscess) and an asymptomatic *C. difficile* fecal excretor had neutralizing IgG as well as neutralizing IgA in their sera. These data characterize a novel functional role for serum IgA distinct from that of IgG in the systemic response to infection with a mucosal pathogen and suggest a selective functional link between systemic IgA and mucosal antigenic challenge.

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