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Further Evidence for Cross-Linking as a Protective Factor in Experimental Cholera: Properties of Antibody Fragments

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Enzymic fragments of IgG antibody were tested for their protective abilities in the infant mouse cholera model. $F(ab')_2$ retained the full protective activity of the parent IgG molecule despite losses in complement fixation and opsonic functions. Fab' and Fab fragments also contained significant protective activity but at a level of only 10% of the intact IgG or $F(ab')_2$. Self-recombinant univalent $F(ab')_2$ also contained about 10% of the protective activity of the divalent $F(ab')_2$ parent molecule. These results are interpreted as evidence that cross-linking is an important mechanism by which specific antibody protects mucosal surfaces against experimental infection with *Vibrio cholerae*.

The infant mouse cholera model described in 1968 by Ujiiye et al. [1] has proved to be a convenient system for investigation of the mechanisms by which local antibody may protect mucosal surfaces against bacterial infections. Studies from this and other laboratories have shown that, after oral infection with *Vibrio cholerae*, infant mice develop typical signs of cholera: multiplication of bacteria in the intestine, severe bowel distension, copious diarrhea, and, finally, death of all unprotected animals [1, 2]. Complete protection, without occurrence of these symptoms, can be achieved either by passive (oral or parenteral) administration of antiserum [2, 3] or by suckling of the infants on mothers specifically immunized with *V. cholerae* [2, 4]. In many respects the protective effects of specific antiserum in this model are similar to the observations by Smith [5] on enteric *Escherichia coli* infections in colostrum-deprived piglets.

We reported that protection against cholera in this model could be mediated equally well by the three major classes of rabbit antibody: IgG, IgM, and secretory IgA [6]. This protective effect was antibacterial, since >80 % of the protective activity was removed by absorption with *V. cholerae* lipopolysaccharide (LPS). The data from this study indicated that, whatever the mechanism of antibacterial protection in this model, it was common to all classes of antibody. Recent evidence from our laboratory [7] has suggested that the mechanism of this common pathway is based on the cross-linking property of specific antibody. In this report we have attempted to verify these findings by a study of the properties of enzymic fragments of rabbit IgG antibody.

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Materials and Methods

Bacterial strains. The strains of *V. cholerae* used in this study were 569B (Inaba) and streptomycin-resistant O17SR (Ogawa) [2, 8, 9]. A hybrid vibrio (569B/165SR) was obtained from Dr. K. Bhaskaran (Central Drug Research Institute, Lucknow, India). This strain contains the H-antigens of 569B, but the O-antigens have been replaced by those of a noncholera vibrio, NCV-165 [10].

Antiserum and purified antibodies. Groups of rabbits were repeatedly immunized parenterally with live strains of *V. cholerae* during periods of two to 12 months, and hyperimmune antiserum was obtained. IgG fractions containing

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activity against *V. cholerae* were purified from individual or pooled antiserum by Sephadex G-200 and DEAE-cellulose chromatography as described previously [6].

A preparation of IgG with activity to *V. cholerae* 569B was used for the isolation of antibody to somatic antigen (anti-O). The IgG preparation was absorbed with a thick suspension of boiled, glutaraldehyde-fixed 569B. After five washing cycles with cold phosphate-buffered saline (PBS, *p*H 7.4), the adsorbed antibody was eluted with 0.2 M glycine/HCl (*p*H 2.2). The eluate was neutralized by dropwise addition of 2 M NaOH and mixed with an approximately equal amount of previously absorbed normal IgG. The solution was then centrifuged at 100,000 *g* for 30 min at 4 C for removal of traces of aggregated protein and antigen-antibody complexes.

A preparation of $F(ab')_2$ containing activity to the O-antigen of *V. cholerae* 569B was prepared so that self-recombinant anti-O univalent $F(ab')_2$ could be studied in the protection experiments. Antibody to flagellar antigen (anti-H) was removed by absorption of the pepsin digest five times with live *V. cholerae* 569B/165SR. After the last absorption the material was centrifuged at 100,000 *g* for 30 min at 4 C and then passed through a column of Sephadex G-200 for purification of $F(ab')_2$. Agglutination tests on the absorbed $F(ab')_2$ against the hybrid strain showed that >99% of the anti-H activity had been removed.

Digestion of IgG with pepsin and papain. Rabbit IgG was digested with pepsin by the method of Nisonoff et al. [11]. Rabbit IgG (5–10 mg/ml) was equilibrated against 0.1 M sodium acetate (*p*H 4.5). Pepsin (twice crystallized, lot no. 69B-20001, Sigma Chemical, St. Louis, Mo.) was then added to the IgG solution; the IgG-to-pepsin weight ratio was approximately 100:1. After incubation at 37 C for 18 hr, the solution was centrifuged for removal of insoluble material, neutralized by dropwise addition of 2 M NaOH, and then dialyzed at 4 C against 2–4 liters of PBS (*p*H 7.4). The dialyzed digest was then fractionated on a column of Sephadex G-200 at 4 C to separate $F(ab')_2$ from intact IgG and fragments of lower molecular weight.

$F(ab')_2$ was reduced (in 0.1 M acetate, *p*H 5.0) to Fab′ with 0.01 M 2-mercaptoethylamine [11]. The Fab′ fragments were then alkylated at 25 C with a 50% molar excess of iodoacetamide; *p*H was maintained between 7 and 8.

Self-recombinant $F(ab')_2$ was prepared from $F(ab')_2$ [12]. The reoxidized material was passed through a Sephadex G-200 column at 4 C for separation of randomly recombined $F(ab')_2$ from Fab′ monomers. Of the eluted protein, 30%–50% reformed into dimeric $F(ab')_2$ by this method; this material is referred to as self-recombinant $F(ab')_2$. The Fab′ monomers eluted from the column were again reduced with 2-mercaptoethylamine, alkylated, and dialyzed against two changes of 0.85% NaCl at 4 C.

Rabbit IgG was digested with papain [13] as follows: IgG (2.5 mg/ml) was dialyzed against 0.05 M sodium acetate (*p*H 5.5) in the presence of 0.002 M EDTA. Cysteine was added to a concentration of 0.05 M; papain (Papaya-Latex, lot no. 01300-2242, twice crystalized in 0.05 M acetate [*p*H 4.5]; Mann Research Labs, New York, N.Y.) was then added to a final concentration of 25 μg/ml. After incubation for 12 hr at 37 C, the digest was dialyzed against PBS (*p*H 7.4) and applied to a column of Sephadex G-200. This step was included to separate Fab/Fc from intact IgG and other divalent material of higher molecular weight. The material obtained in this way was called purified papain digest.

Preparations of IgG and fragments were finally dialyzed against 0.85% NaCl at 4 C and stored in small aliquots at −15 C. Protection experiments on these materials were performed one to two weeks after preparation.

Determination of protein. The protein content of purified IgG and fragment preparations was determined from the optical density (OD) at 280 μm; extinction coefficients used were 13.6 for IgG [14], 14.8 for $F(ab')_2$ [15], 14.8 for Fab′, and 13.6 for the purified papain digest.

Quantitative precipitin tests. The amount of antibody to purified LPS of *V. cholerae* 569B was determined by quantitative precipitin tests [8].

HA tests. The preparation of purified *V. cholerae* LPS and the sensitization of sheep red blood cells (SRBC) with alkali-treated LPS have been described [8]. LPS-coated SRBC (at 1% vol/vol) were added to serial twofold dilutions (in NaCl) of IgG or fragment preparations. End

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points were read after incubation for 1 hr at 37 C and for another 3–16 hr at room temperature (about 24 C). For tests of the effect of the reducing agent, 2-mercaptoethanol (2-ME), on HA activity, the material tested was preincubated with 0.1 M 2-ME in PBS (*p*H 7.4) for 60 min at 37 C before serial dilution and addition of LPS-SRBC. Approximately 1.0 μg of IgG antibody directed to LPS/ml was detected by the HA assay [6].

Tube agglutination tests. Serial dilutions of antibody were made in PBS (*p*H 7.8). An equal volume (0.5 ml) containing 5×10^9 twice-washed live *V. cholerae* was added to each tube. Tubes were incubated at 37 C for 1–2 hr, and macroscopic agglutination end points were read after incubation for 4–16 hr at 4 C.

Hemolytic tests. For complement-dependent passive hemolysis, fresh guinea pig serum was added to a final dilution of 1:40–1:80 to the settled LPS-SRBC patterns in the HA test. End points were read after incubation for 1–2 hr at 37 C.

Vibriocidal tests. This assay has been described previously [8, 16]. Antibody and fragment preparations were serially diluted (twofold steps) in 0.1% (wt/vol) proteose-peptone in NaCl. Log-phase broth cultures of *V. cholerae* were diluted to give $4–10 \times 10^3$ viable bacteria/ml of guinea pig serum diluted 1:10 in proteose-peptone-NaCl. Equal volumes (0.5 ml) of bacteria in guinea pig serum and of the antibody dilution were mixed and incubated at 37 C for 60 min before plating of 0.1 ml onto nutrient agar. The end point was taken as that dilution resulting in killing of 50% of the bacteria in 60 min.

Tests of opsonic activity. The opsonic activity of a given preparation was determined in vivo by the ip test in adult mice [17]. The end point was defined as that dilution giving 50% clearance of *V. cholerae* at 60 min compared with an unopsonized control [6]. The mice used for these tests were either outbred or C57BL and weighed 20–22 g. Both strains had similar end points for a given preparation.

Protection tests in infant mice. This test has been described in detail elsewhere [1–3]. In brief, five- to six-day-old outbred mice were separated from their mothers and kept in tissue-lined containers in the laboratory. The protective efficiency of a preparation was determined by pretreatment of 10–1,000 LD_{50} of a given bacterial strain with serial 10-fold dilutions of test material in NaCl and oral administration of 0.1 ml to each mouse. The controls in all experiments were groups of mice given normal rabbit serum (diluted 1:10–1:100) together with the challenge organisms. The dilution of test material necessary for protection of 50% of the mice (PD_{50}) was calculated at a time when all control mice were dead [18]. The average time to death for the controls in this study was usually 48 hr. Untreated or completely protected mice survived under these conditions for at least 72 hr.

The mixture of antibody with vibrios at the time of challenge is convenient and ensures maximal sensitivity in the protection test. Significant protection was obtained, however, if antibody was administered either orally or systemically several hours before challenge (authors' unpublished observation).

Results

Purity of F(ab')$_2$ preparations. The F(ab')$_2$ preparations described in this report contained little, if any, intact IgG.[1] The residual vibriocidal and opsonic activities found in all F(ab')$_2$ fractions studied (table 1) were intrinsically associated with F(ab')$_2$ and could not be explained by contamination with intact parent IgG.[2]

Biological properties of fragment preparations. The properties of various fragment preparations are shown in table 1. F(ab')$_2$ has clearly reduced complement-dependent functions in vitro (hemolysis, vibriocidal activity) and opsonic functions in vivo, yet retains both the cross-linking (HA) and the protective (PD_{50}) activity of the parent IgG molecule. The loss of complement-related functions and opsonic activity is essentially consistent with all available evidence in the literature on the biological properties of rabbit F(ab')$_2$ antibody [19–24]. The results in table 1 support the earlier conclusion [6] that the protection of infant mice from experimental cholera is not related to the vibriocidal or

[1] E. J. Steele and D. Rowley, "The Mechanism of Complement Fixation by Rabbit F(ab')$_2$," manuscript in preparation.

[2] See footnote 1.

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Table 1. Biological properties of IgG, $F(ab')_2$, and Fab′ containing antibody activity to live *Vibrio cholerae*.

Experiment no.	Preparation	Bacterial strain	Activity* HA	HL	Vib	Op	PD_{50}
1	IgG	569B	0.7	1.6	160	340	5.0
	$F(ab')_2$		1.3	0.002	16	28	12.5
2	IgG		1.0	2.2	40	ND†	3.0
	$F(ab')_2$	569B	0.9	0.002	9	ND	3.0
	Fab′		≤0.002	<0.002	0.6	ND	0.2
3	IgG		0.5	0.9	550	3,800	0.5
	$F(ab')_2$	O17SR	0.5	0.003	45	360	0.5
	Fab′		<0.01	<0.01	0.2	5	0.03

* HA = hemagglutination; HL = hemolysis; Vib = vibriocidal activity; Op = opsonic activity; PD_{50} = protection (in terms of doses protecting 50% of infant mice). Levels of activity are levels per μg of antibody to *V. cholerae* lipopolysaccharide and are reciprocal end-point dilutions in a given assay.

† ND = not determined.

opsonic capacity of the antibody preparation used.

It is also evident from table 1 that the reduction of $F(ab')_2$ to Fab′ causes a further loss in vibriocidal and opsonic activity and a complete loss of the cross-linking function. The protective activity of these monomeric fragments is also reduced (by approximately 10-fold) from the level with $F(ab')_2$. The observed protection with Fab′ could theoretically be due to residual divalent $F(ab')_2$ (or traces of intact IgG). In experiment no. 2 (table 1) this theory might be valid, since the residual vibriocidal activity [a measure of $F(ab')_2$ or IgG] correlates with the observed level of protection in Fab′; the same does not hold for experiment no. 3.

Properties of a self-recombinant $F(ab')_2$ molecule. The data in table 1 provide strong presumptive evidence that divalency or cross-linking ability of specific antibody is necessary for full expression of protection in the infant mouse cholera model. A more direct demonstration of this fact is shown in table 2, in which a recombinant $F(ab')_2$ fragment shows a 14-fold decrease in protective ability and an equal or greater reduction in all of those activities dependent on a bivalent $F(ab')_2$ molecule.[3]

Protective activity of Fab′ fragments. Is the residual protective activity associated with Fab′ due to contamination with a divalent cross-linking moiety, or does it reflect a true property of univalent monomers? To eliminate the possibility of reassociation through unblocked cysteine residues, rabbit IgG was digested with papain, and the digest was fractionated on Sephadex G-200 to separate Fab/Fc from higher molecular weight (dimeric?) fragments and intact IgG. The data in table 3 show that a purified digest (Fab/Fc) has <0.2% of the microscopic agglutinating ability of intact IgG, yet has significant (12.5%) protective activity. Univalent fragments appear to have some protective value but at a level 10 times less efficient than either IgG or $F(ab')_2$.

[3] In the context of table 2, the vibriocidal activity may be considered a measure of divalency in $F(ab')_2$. Full expression of complement-dependent functions in $F(ab')_2$ requires that the specific Fab′ subunits are arranged symmetrically in the $F(ab')_2$ dimer. See footnote 1.

Discussion

In a previous study [6] we showed that the protective effect of specific antibody against *V.*

Table 2. Demonstration that cross-linking by specific $F(ab')_2$ is required for efficient protection against infection with *Vibrio cholerae* 569B in infant mice.

Preparation	Activity* HA	Vib	Agg	PD_{50}
$F(ab')_2$	3.3	140	1.0	13
$F(ab')_2$ (self-recombinant)	0.08	4.8	<0.08	0.9
Fab′	<0.02	<0.2	<0.1	0.8

* HA = hemagglutination; Vib = vibriocidal activity; Agg = agglutination of live organisms; PD_{50} = protection (in terms of doses protecting 50% of infant mice). Levels of activity are levels per μg of antibody to *V. cholerae* lipopolysaccharide and are reciprocal end-point dilutions in a given assay.

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Table 3. Protective activity of a purified papain digest.

	Activity*				
			Agg		
Preparation	HA	Vib	Macro	Micro	PD_{50}
IgG	1.5	100	0.1	3.6	0.64
Purified digest (Fab + Fc)	<0.003	<0.1	<0.007	<0.007	0.08

NOTE. See Materials and Methods for details on preparation of purified papain digest.

* HA = hemagglutination; Vib = vibriocidal activity; Agg = agglutination of live organisms; Macro = macroscopic (determined with use of 5×10^9 live *Vibrio cholerae* 569B/ml in a tube agglutination test); Micro = microscopic (assessed on stained smear [$1–2 \times 10^8$/ml] before oral administration to infant mice); PD_{50} = protection (in terms of doses protecting 50% of infant mice). Levels of activity are levels per μg of antibody to *V. cholerae* lipopolysaccharide and are reciprocal end-point dilutions in a given assay.

cholerae in infant mice was unrelated to CF or opsonic functions. We predicted that the protective mechanism (common to IgG, IgM, and secretory IgA) was independent of functional variations in the Fc region of the antibody molecule. The results in this paper unequivocally support this speculation. The data show that $F(ab')_2$ fragments, which do not contain the classical Fc region, have undiminished protective ability in the infant mouse cholera model.

Recent studies from our laboratory [7] have suggested that agglutination and prevention of adherence of *V. cholerae* to epithelial cells are significantly correlated with protection. Anti-H was shown to mediate efficient protection; presumably anti-H could function only by the mechanical effects of agglutination or inhibition of motility. The data presented here support the idea that physical cross-linking by divalent antibody is all that is required for full expression of the protective effect (table 2).

The available evidence (reviewed in [25]) indicates that adherence of bacteria to the epithelium is a prerequisite for colonization of mucosal surfaces. The data in this paper and elsewhere [7] are consistent with the idea that antibody protects against intestinal cholera infections by reducing adsorption of vibrios to the intestinal epithelium. The mechanism of this reduced adsorption is mediated principally by the cross-linking function of specific antibody.

Since univalent Fab', Fab, or $F(ab')_2$ fragments show decreased but significant protection, simple coating of the bacteria may also reduce association with the epithelium, either by masking or by interfering with adhesive receptors on the bacterial surface.

In summary, we consider that the cross-linking of any available bacterial antigens (O, H, or an undefined type) by antibody leads to a reduced association between the bacteria and the intestinal epithelium [7] and (in conjunction with peristalsis [26]) results in the efficient elimination of bacteria from the small intestine. These conclusions are entirely consistent with those of Freter [27] and of Williams and Gibbons [28] on the ways in which antibacterial antibody may protect mucosal surfaces against superficial bacterial infections.

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