

Dog Immunoglobulins

II. THE ANTIBACTERIAL PROPERTIES OF DOG IgA, IgM AND IgG ANTIBODIES TO *VIBRIO CHOLERAE*

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Summary. Secretory IgA antibodies to *Vibrio cholerae* were purified from the parotid saliva and mammary secretions of locally and orally immunized dogs using gel filtration, ion-exchange chromatography and anti-immunoglobulin immunoabsorbents. IgM and IgG antibodies were isolated from serum by gel filtration and ion-exchange chromatography.

IgA antibodies proved to have minimal, if any, activity in direct killing of bacteria in the presence of complement or in the promotion of phagocytosis. The minimal activity which IgA had in these assays could be accounted for by extremely small quantities of IgM antibody. The same IgA antibodies, mixed with the challenge inoculum of *Vibrio cholerae* and fed to infant mice, protected these mice as efficiently as IgG or IgM antibodies.

INTRODUCTION

Although Heremans, Heremans and Schultze first isolated IgA in 1958, the role that this and other immunoglobulins play at mucous surfaces remains unclear. In particular, there are conflicting reports regarding the anti-bacterial ability of secretory IgA.

It has been established that IgG and IgM antibodies are able to promote killing of some Gram-negative bacteria by (a) *in vitro* killing in the presence of complement and (b) promotion of phagocytosis (Robbins, Kenny and Suter, 1965; Eddie, Schulkind and Robbins, 1971; Knop, Breu, Wernet and Rowley, 1971). IgA antibodies do not mediate complement-dependent cell lysis (Ishizaka, Ishizaka, Borsos and Rapp, 1966; Adinolfi, Mollison, Polley and Rose, 1966) but Adinolfi, Glynn, Lindsay and Milne (1966), and more recently Hill and Porter (1974), have reported that specific secretory IgA causes lysis of *E. coli* only in the presence of both lysozyme and complement. Eddie *et al.* (1971) did not observe any bactericidal effect of purified IgA antibodies against *Salmonella typhimurium* in the presence of both complement and lysozyme and, with *Vibrio cholerae* and *E. coli*, we have been unable to establish, irrespective of the presence of lysozyme, any *in vitro* killing activity for secretory IgA antibodies from several species (Heddle, Knop, Steele and Rowley, 1975).

Some studies have suggested that secretory IgA antibodies are efficient opsonins (Knop *et al.*, 1971; Kaplan, Dalmaso and Wilson, 1972), but Quie, Messner and Williams

(1968), Eddie *et al.* (1971), Wilson (1972) and Steele, Chaicumpa and Rowley (1974) have all reported absent or minimal activity for secretory IgA antibodies in promoting phagocytosis of bacteria.

We have tested dog IgA, IgM and IgG antibodies to *Vibrio cholerae* for their capacities to kill that organism *in vitro* in the presence of complement, and for their abilities to promote phagocytosis using the mouse intraperitoneal opsonic model (Whitby and Rowley, 1959). The protective capacities of the same materials were also tested in the infant mouse cholera model (Ujjiye, Nakatomi, Utsunomiya, Mitsui, Sogami, Iwanaga and Kobari, 1968). Our results confirm the lack of *in vitro* and opsonic activities of specific antibody of the IgA class but show that in the *in vivo* infant mouse cholera model it has similar efficiency to the other two classes.

MATERIALS AND METHODS

Animals

Dogs and goats were selected as described in the accompanying paper (Heddle and Rowley, 1975).

Immunization, sample collection and storage

Immunization and the storage of samples have been described (Heddle and Rowley, 1975). Colostrum and milk were obtained from dogs which had received either (a) 7 g of sodium bicarbonate orally followed by 2×10^{10} live thrice-washed *Vibrio cholerae* 569B in a gelatin capsule 16 and 13 days before parturition, or (b) perimammary injections of 5×10^9 washed, 56° killed, *Vibrio cholerae* 569B and four to ten oral doses of about 10^{11} of the same organisms over the month prior to parturition.

Samples which had been stored with sodium azide were dialysed exhaustively against azide-free phosphate-buffered saline before use.

Fractionation of immunoglobulins

Gel filtration. This was carried out using Sephadex G-200 and Sepharose 6-B (Heddle and Rowley, 1975).

Ion-exchange chromatography. This employed DEAE-cellulose (DE-52) and sodium phosphate buffer (0.01–0.50 M) pH 8.0 containing 0.02 per cent sodium azide.

Affinity chromatography. Globulin preparations from goat anti-dog immunoglobulin sera were absorbed with insolubilized immunoglobulins to remove light chain specificities (Heddle and Rowley, 1975). They were then concentrated to the volumes of the sera from which they had been derived and their heavy chain specificities verified (Heddle and Rowley, 1975). Twenty millilitres of each globulin solution was covalently linked to 20 ml of Bio-Gel A-50 (Heddle and Rowley, 1975). The anti- α , anti- μ and anti- γ columns could bind respectively 97, 95 and more than 99 per cent of a 5 mg load of the appropriate immunoglobulin. After washing the columns with phosphate-buffered saline, pH 7.1, the absorbed immunoglobulins were eluted with similarly buffered 3.0 M sodium thiocyanate (Bennich and Johansson, 1971). Buffered 3.5 M sodium thiocyanate was passed through the columns before re-use.

Antisera and immunochemical assays

The anti-immunoglobulin sera and immunochemical techniques used have been

described (Heddle and Rowley, 1975). The preparation of immunoglobulin standards is described in this paper. Optical densities of protein solutions were determined as described by Heddle and Rowley (1975). A goat anti-dog colostrum serum and rabbit antisera to dog colostrum and serum were raised in this laboratory. They were used in conjunction with a commercial rabbit antiserum to dog serum (Miles Laboratories).

Assays for antibody to Vibrio cholerae 569B lipopolysaccharide (569B-LPS)

Vibrio cholerae 569B (Inaba serotype, 'Classical' biotype). This was passaged and maintained in this laboratory, as described by Neoh and Rowley (1970).

569B-LPS. This was extracted and prepared for sensitization of sheep red blood cells (SRBC) using published techniques (Steele *et al.*, 1974).

Passive haemagglutination assay (LPS-HA). SRBC were sensitized with 569B-LPS and used as described by Auzins (1968). Washed unsensitized SRBC were included as controls.

Antiglobulin enhanced passive haemagglutination assays ('Coombs' tests) used a published modification (Steele *et al.*, 1974) of the assay described by Coombs, Gleeson-White and Hall (1951). LPS-sensitized and normal SRBC (controls) were incubated with antibody dilutions, washed three times in ice-cold saline, resuspended to 1 per cent and mixed with equal volumes of saline or of antisera to dog α , μ or γ chains at dilutions previously found to provide optimal enhancement of agglutination (approximately 1 in 1,000). Following further incubation the results were read as for LPS-HA.

Direct determinations of specific anti-569B-LPS antibody. These were performed as described by Steele *et al.* (1974).

569B-LPS absorptions of antibody preparations. The 569B-LPS, antibody mixture and a control lacking lipopolysaccharide were incubated at 37° for 1 hour, 4° for 40 hours and then centrifuged at 80,000 *g* for 90 minutes.

2-Mercaptoethanol treatment of antibody. This involved mixing antibody with an equal volume of 0.2 M mercaptoethanol, freshly diluted in phosphate-buffered saline, pH 7.4, and incubating for 1 hour at 37°. Samples were dialysed free of mercaptoethanol before use in biological assays.

'Vibriocidal' assay. This *in vitro* technique was described by Neoh and Rowley (1970). The end point was taken as the reciprocal of the greatest dilution of antibody that provided greater than 90 per cent killing of *Vibrio cholerae 569B* over 90 minutes at 37°. Complement controls lacking added antibody were always employed and controls containing antibody but not complement were included until it became clear that antibody alone, whatever the immunoglobulin class, did not reduce viable counts. In order to remove any 'prozone' effects, all materials were tested up to dilutions 100 times greater than the end point.

'Opsonic' assay. The model of Whitby and Rowley (1959) was used to study phagocytosis of *Vibrio cholerae 569B* in the peritoneal cavities of normal young adult mice. The end point was the reciprocal of the antibody dilution (by interpolation) that achieved 90 per cent fall in counts relative to an unopsonized (saline) control over 45 minutes.

In vivo protection of infant mice challenged orally with Vibrio cholerae 569B ('protection' assay). This 'cholera' model was described originally by Ujiye *et al.* (1968). Five to 6-day-old mice were separated from their mothers for 2 hours and then fed 2×10^7 log phase *Vibrio cholerae 569B* (about 10 LD₅₀) in 0.1 ml. This inoculum had been pretreated at room temperature for 20 minutes either with heat-inactivated normal serum diluted 1 in 100 or with a dilution of antibody. For each antibody or control dilution, eight to fifteen mice were challenged and the reciprocal of the dilution giving 50 per cent survival, at a time when the

last normal serum-treated mouse had just died, was calculated by the method of Reed and Muench (1938). 569-LPS absorptions of antibody were performed as above and used approximately 10 μ g of 569B-LPS per microgram of anti-569B-LPS antibody.

RESULTS

PURIFICATION OF DOG ANTIBODIES TO *V. cholerae*

Fractionation procedures

Fractionation of dog immunoglobulins on Sephadex G-200 was described in the companion paper (Heddle and Rowley, 1975). Gel filtration on Sepharose 6-B discriminated more usefully between secretory IgA and IgM (Fig. 1). Ion-exchange chromatography of secretory IgA and IgM on DE-52 using a linear gradient of sodium phosphate buffer pH 8.0 is illustrated in Fig. 2. IgG eluted at lower molarities of the same buffer. When the

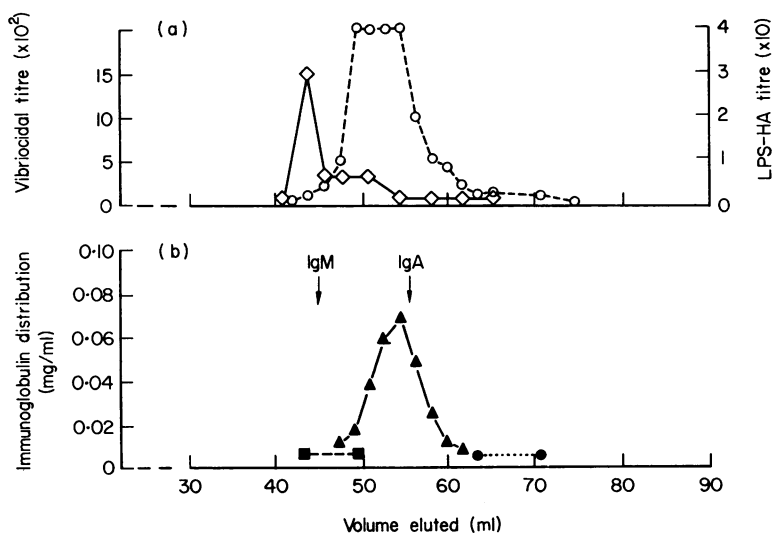


FIG. 1. Gel filtration of anti-*V. cholerae* parotid saliva on Sepharose 6-B. Although IgM and IgG were detected clearly over the fractions shown, it was not possible to quantificate them accurately. The elution peaks from fractionation of IgM and secretory IgA on the same column are indicated. (a) Antibody activities. (\diamond) vibriocidal titre; (\circ) LPS-HA titre. (b) Immunoglobulin distribution: (\blacktriangle — \blacktriangle) IgA; (\blacksquare — \blacksquare) IgM (traces only); (\bullet ... \bullet) IgG.

IgG fraction from a Sephadex G-200 filtration of blood serum was dialysed against 0.01 M buffer and eluted from a DE-52 column in batches of 0.01, 0.04, 0.08 and 0.50 M buffer, 60 per cent of the IgG eluted at a molarity of 0.01, a further 30 per cent at 0.04 and all but 1 per cent had eluted at the conclusion of the 0.08 M column wash. Immunoelectrophoresis (IE) of the 0.01 M eluate at 3 mg/ml against antisera to dog serum revealed only IgG2a, b (Johnson and Vaughan, 1967). Double immunodiffusion against reference antisera to dog γ 1 and γ 2 (kind gifts of Professor J. F. Heremans and Dr J.-P. Vaerman (Heddle and Rowley, 1975)) revealed only IgG2. The 0.04 M eluate contained appreciable quantities of IgG1.

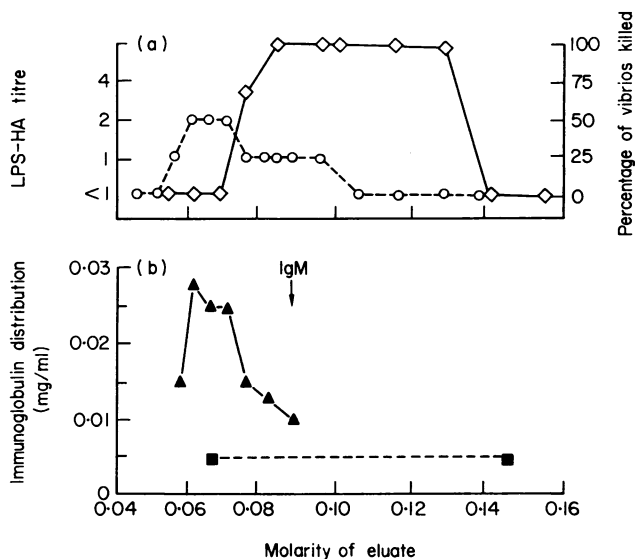


FIG. 2. Ion-exchange fractionation of IgA antibody to *V. cholerae*. As a final step in purification, IgA (batch I, J) was eluted from a DE-52 column using a gradient of sodium phosphate buffer, pH 8.0. Because of the low levels remaining, vibriocidal activities are indicated as the percentages of vibrios killed by 1:2 dilutions of dialysed fractions. The elution profile for IgM under the same conditions is shown. (a) Antibody activities: (○) LPS-HA titre; (◇) percentage of vibrios killed. (b) Immunoglobulin distribution: (▲—▲) IgA; (■--■) IgM.

IgG and IgM antibodies

Table 1 lists the sources, the preparatory steps and the results of single radial immunodiffusion (SRID) analyses with anti-immunoglobulin heavy chain sera for the IgG and IgM standards. Some properties of the IgG preparation have been described above, notably that on IE at 3 mg/ml against antisera to dog serum no impurities were detected. All other IgG preparations were isolated by the same procedure and consistently gave similar results on SRID analysis.

The IgM standard on IE at 2 mg/ml against antisera to dog serum yielded only an IgM precipitin line. Gel filtration of this IgM on a Sepharose 6-B column revealed a single macromolecular optical density peak symmetrical with anti- μ reactivity in SRID but in extrapolating optical density measurements on the standard to concentrations of IgM

TABLE 1
DOG IgG AND IgM STANDARDS

Immunoglobulin	Source	Fractionation procedures	SRID analysis*
IgG	Serum	Sephadex G-200 and 0.01 M eluate from DE-52	<0.2 IgA <0.2 IgM
IgM	Serum	Sephadex G-200 and 0.09–0.15 M eluate from DE-52	2 IgA <0.7 IgG

* Proportion of total immunoglobulin by weight, expressed as a percentage.

(mg/ml) (Heddle and Rowley, 1975), correction had to be made for the optical densities of small molecular weight materials that eluted near the void volume. A few other IgM samples, prepared by a similar technique, showed a faint precipitin line of α mobility on IE against antisera to dog serum. Contamination of IgM by IgA and IgG never exceeded a total of 2.5 per cent. Tests for mercaptoethanol sensitivities and antiglobulin enhancement of antibody activities (Table 2) confirmed that the anti-569B-LPS activities of IgG and IgM preparations resided in the respective immunochemically dominant classes.

TABLE 2
PURIFIED DOG IgG AND IgM ANTI-*Vibrio cholerae* ANTIBODIES

Immunoglobulin class	Coombs test*				Percentage mercaptoethanol resistance	
	Anti- α	Anti- μ	Anti- γ	n	LPS-HA	Vibriocidal†
IgG	1	1	26	7	100 (n = 4)	100 (n = 2)
IgM	1	4	1	4	2 (n = 2)	2 (n = 2)

* Expressed as anti-dog immunoglobulin heavy chain enhanced titre: saline titre. Values represent geometric means of 'n' tests.

† Absorption with 569B-LPS reduced the original activities by 90 per cent or more.

Secretory IgA antibodies

As assessed by Coombs' tests (compare section on 'estimation of anti-569B-LPS antibody content'), the starting materials contained a majority of the antibody to 569B-LPS in IgA (Table 3). The isolation from colostrum of IgA batch F, used in the IgA standard (F, H), is illustrated (Table 4). The usefulness of following LPS-HA and vibriocidal activities during purification is evident.

The majority of IgG was removed from IgA on passage of colostrum through Sephadex G-200, whereas the IgM and most of the remaining IgG were removed on anti- μ and anti- γ columns. The immunoglobulin fractions obtained were pooled with the respective fractions from a parallel run H, concentrated and analysed. Twenty to 30 per cent of the

TABLE 3
ANTIGLOBULIN-ENHANCED PASSIVE HAEMAGGLUTINATION TITRES OF IgA SOURCES (COOMBS' TEST)

IgA source	Sheep red blood cells	Coombs' test titre*			
		Anti- α	Anti- μ	Anti- γ	Saline
Parotid saliva	569B-LPS†	400	300	≤ 100 ‡	≤ 100 ‡
Mammary secretion	Normal	5	5	4	4
	569B-LPS	100	40	40	30

* Reciprocal of end point dilution of saliva or mammary secretion. Values are geometric means.

† Sheep red blood cells were sensitized with *V. cholerae* 569B lipopolysaccharide. Normal sheep red blood cells were run in parallel but did not agglutinate at the dilutions of saliva tested.

‡ Maximum possible values.

TABLE 4
PREPARATION OF SECRETORY IgA (BATCH F)

Sample	Immunoglobulin content (mg) determined by SRID			LPS-HA (titre* × volume)	Vibriocidal (titre* × volume)
	IgA	IgM	IgG		
Day I, II colostrum	23	2.6	160	1000	30,000
Interim IgA-I	15	2.7	4	600	40,000
Interim IgA-II	4.7	<0.14	<0.23	100	300
IgA-Batch F	3.2	<0.04	0.08	100	80

* Reciprocal of greatest effective dilution.

vibriocidal and LPS-HA activities were recovered, but while 50 per cent of the recovered LPS-HA activities lay in IgA (F, H) all but 0.2 per cent of the recovered vibriocidal activities lay in the IgG (20 per cent) and IgM (80 per cent) fractions (respectively, the combined IgG peaks from Sephadex G-200 fractionations and anti- γ eluates and the anti- μ eluates). On IE at 2 mg/ml against antisera to dog colostrum, this IgA standard revealed only a single IgA precipitin line. Fractionation on Sepharose 6-B yielded a single macromolecular optical density peak coincident with the anti- α reactivity in SRID and, as had been observed with the IgM standard, a small optical density peak in fractions eluting near the void volume. Correction was made for the optical densities of these low molecular weight materials in calculating the IgA content of the purified standard from its optical density. Before this standard had been prepared, dilutions of partially purified colostrum IgA and of a dog serum pool had been used in SRID with anti- α chain serum. By titrating the serum dilutions in SRID (under uniform conditions) against dilutions of the definitive standard, a continuity of results was obtained.

The methods by which other IgA batches were prepared, and some criteria for their purity, are shown (Table 5). A few of the procedures (A and C) were designed solely to remove IgG and IgM from IgA and would not have removed other macromolecules. Knowing the vibriocidal activities and IgM concentrations of the 'IgM' fractions isolated as 'by products' of IgA purification, estimates were made of the IgM content of IgA preparations.

ACTIVITIES OF DOG ANTIBODIES TO *Vibrio cholerae*

Estimation of anti-569B-LPS antibody content

To compare the activities of the different immunoglobulins, it was necessary to know the quantities of antibody to 569B-LPS present in each preparation. Because of the quantities available and their activity, it was not possible to measure this directly with purified dog IgA or IgG antibodies. With E. Steele (Steele *et al.*, 1974) we were able to demonstrate that when antibody to 569B-LPS in rabbit immunoglobulins and dog IgM was measured directly, the results correlated extremely well with Coombs' test titres, irrespective of the immunoglobulin class. For example, rabbit IgG, IgM and IgA antibodies (Steele *et al.*,

TABLE 5
SECRETORY IgA PREPARATIONS

Batch	Source(s)	Purification procedures*	Immunoglobulin impurities†		
			SRID		Vibriocidal IgM
			IgM	IgG	
A	Saliva	A μ , A γ	< 6	< 8	3
B	Saliva	M1, A μ , A γ , A α	< 5	< 7	< 1
C	Saliva	M1, A μ , A γ	< 2	< 4	0.4
E‡	Milk	A μ , A γ , A α	< 2	< 1	1
F, H	Colostrum and milk	M1, A μ , A γ , I	< 0.4	1	0.1
I, J	Milk and saliva	M1, M2, I	< 3	< 3	0.3

* The symbols represent gel filtration on Sephadex G-200 (M1) and Sepharose 6-B (M2), ion-exchange chromatography (I) and affinity chromatography (A μ , A γ , A α).

† Impurities are expressed as percentage by weight of total immunoglobulin, as estimated by SRID and vibriocidal assays (see text).

‡ IgA batch D was without relevant specific antibody.

1974) gave Coombs' titres, when enhanced with antisera to the respective rabbit immunoglobulin heavy chains, of 36, 38 and 28 for each microgram of directly determined anti-569B-LPS antibody per millilitre. The corresponding value for dog IgM antibody was 33. In the Coombs' test, titres of dog IgG and IgA antibody preparations were enhanced 26 and 7 times with antisera to the respective dog heavy chains over those with saline. These enhancements are inversely proportional to the known agglutination efficiencies per unit weight of specific antibody for these classes in the rabbit (Eddie *et al.*, 1971; Steele *et al.*, 1974). Therefore it seems reasonable to accept the heavy chain-specific Coombs' titres as being proportional to the specific antibody content (by weight) of each class.

Activities in 'vibriocidal', 'opsonic' and 'protection' assays

IgM antibodies proved to be extremely efficient at promoting *in vitro* complement mediated killing and phagocytic killing in the mouse peritoneum when compared to IgG and particularly, IgA antibodies in terms of limiting antibody dilutions at which solutions estimated to contain equivalent amounts of antibody to 569B-LPS remained

TABLE 6
VIBRIOCIDAL AND OPSONIC ACTIVITIES OF DOG IgG, IgM AND IgA ANTIBODIES TO *V. cholerae* 569B-LPS

Immunoglobulin	Vibriocidal		Opsonic	
	Number of times tested	Mean \pm s.e.m.	Number of times tested	Geometric mean (range)
IgG	8	4 \pm 2	3	0.6 (0.4-2)
IgM	12	110 \pm 30	3	10 (6-20)
IgA	9 (six IgA preparations)	\leq 0.3 \pm 0.2*	4 (three IgA preparations)	0.04 (0.03-0.06)

Values given are activities per specifically enhanced passive haemagglutination unit (Coombs' test).

Absorption of IgG and IgM antibodies with 569B-LPS removed more than 97 per cent of their vibriocidal and opsonic activities.

* Maximum possible value.

effective (Table 6). The remarkably high activities of IgM antibodies could be removed by absorptions with 569B-LPS against which the less efficient immunoglobulins had comparable amounts of specific antibody.

Despite these differences, IgA (and IgG) antibodies to 569B-LPS were remarkably effective at protecting infant mice from oral challenges with *Vibrio cholerae* 569B (Table 7).

TABLE 7
INFANT MOUSE CHOLERA MODEL. PROTECTIVE ACTIVITIES OF DOG IgG, IgM
AND IgA ANTIBODIES TO *V. cholerae* 569B-LPS

Immunoglobulin	Number of times tested	Geometric mean	Standard error (range)
IgG	9	0.7	0.1
Activity remaining after 569B-LPS*	6	<0.2	(<0.05-0.6)
IgM	6	0.08	0.03
Activity remaining after 569B-LPS	5	<0.02	(<0.01-0.03)
IgA†	11	0.3	0.06
Activity remaining after 569B-LPS	4	<0.07	(all <0.1)

Values given are activities (reciprocals of antibody dilutions giving 50 per cent protection) per specifically enhanced passive haemagglutination unit (Coombs' test).

* Absorbed with 569B-LPS.

† IgA results are the means of eleven tests on six separate preparations.

DISCUSSION

We have paid constant attention to ensuring that the antibody activities measured resided in the dominant immunoglobulin in each preparation. All IgM samples tested for biological efficiencies (Tables 6 and 7) were isolated from sera that contained most of the antibody to 569B-LPS in the IgM class. In making this statement, account was taken of the higher agglutination efficiency of IgM (Pike, 1967). IgM preparations enhanced in the Coombs' test only with anti- μ , had their anti-569B-LPS activities markedly reduced by mercaptoethanol (Table 2) and had high antibody activities in those assays in which major class differences were evident (Table 6). Clearly the activities attributed to 'IgM' reflected IgM antibody activity.

Contamination of IgG preparations by IgA or IgM was not observed by a SRID technique that would have revealed 0.2 per cent of either of these proteins (Table 1). As with IgM, tests for antibody activities (Table 2) indicated that the antibody to 569B-LPS in the IgG preparations was indeed IgG. We consider that the reported 'IgG' activities reflect properties of IgG2 and probably of the IgG2a,b subclass.

The value of following biological markers during purification procedures was illustrated in studying IgA. Most or all vibriocidal activity paralleled IgM concentrations during preparative steps, as illustrated in isolation steps for IgA batches F (Table 4) and I and J (Figs 1 and 2). Therefore, calculations were made of IgM contaminations of IgA based on vibriocidal activities (Table 5), which, by assuming that all vibriocidal activity in IgA resulted from IgM, maximized the contamination. Frequently these calculations detected 'impurities' not demonstrable by anti- μ SRID analyses. We were never able (Table 5) to detect vibriocidal activity in IgA that could not be accounted for by quantities of the

IgM isolated concurrently, that were smaller than those which could be excluded by SRID.

We regard the demonstrably low IgM contaminations of IgA (Table 5) as indicative of extremely low levels of IgG impurities because, in the ion-exchange and gel filtration fractionations, IgM eluted nearer secretory IgA than did IgG (Figs 1 and 2) (Heddle and Rowley, 1975). The anti- γ immunoabsorbent column had a higher capacity than the anti- μ column. IgA batch F was prepared from early colostrum (Tables 4 and 5) which had, unlike all other IgA sources used, a high IgG content (Heddle and Rowley, 1975). This rendered it suitable to illustrate purification procedures (Table 4) and explains the small contamination with IgG (Table 5).

We believe that the estimates of specific antibody content based upon Coombs' test titres represent a reasonable approach to the problem that confronted us. The specifically enhanced titres correlated well with those direct measurements of weight per millilitre of anti-569B-LPS antibody we were able to make.

Our failure to establish activity of IgA antibodies in *in vitro* killing of *Vibrio cholerae* in the presence of complement (Table 6) is consistent with the results of a majority of authors working with this and other Gram-negative organisms (Eddie *et al.*, 1971; Knop *et al.*, 1971; Fubara and Freter, 1973; Steele *et al.*, 1974). The presence of both lysozyme and complement (Adinolfi *et al.*, 1966) does not confer significant vibriocidal activity on dog IgA antibodies to *Vibrio cholerae* 569B (Heddle *et al.*, 1975).

Our results (Table 6) do not support claims that IgA antibodies are powerful opsonins (Knop *et al.*, 1971; Kaplan *et al.*, 1972), although these authors used a different bacterial strain of red cells. It is questionable, especially in view of the probability that the vibriocidal activities of our IgA preparations represented contaminating IgM antibodies and the similarity of the vibriocidal:opsonic activity ratios for IgA and IgM (Table 6), whether our IgA antibodies had any opsonic activity. Certainly, far more IgA than IgG or IgM anti-569B-LPS antibody was required to achieve the same degree of opsonization. That the killing of Gram-negative bacteria, including *Vibrio cholerae* (Jenkin and Rowley, 1960), in the opsonic model is macrophage-associated has been established (Whitby and Rowley, 1959). Our findings on the opsonic efficiencies of IgA, IgM and IgG antibodies to Gram-negative bacteria resemble closely those of Steele *et al.* (1974) and of Eddie *et al.* (1971), although the latter used a different opsonic model. Using human red cells in an *in vitro* phagocytic system, Zipursky, Brown and Bienenstock (1923) established that any opsonic activity in their IgA was the result of contaminating, highly active, IgM antibody.

Infant mice challenged orally with live *Vibrio cholerae* 569B develop a disease that resembles human cholera in some important respects. The challenge organisms multiply in the lumen of the intestine, the animals develop diarrhoea and at autopsy have distended intestines filled with clear fluid (Ujjiye *et al.*, 1968). At autopsy, although *Vibrio cholerae* can be recovered quantitatively from homogenized infant mice (Chaicumpa, 1974), all but 0.002 per cent of viable organisms were found in the intestine (Chaicumpa, 1974) indicating that this infection, like that in the human, is localized.

There was no correlation between the activities of different immunoglobulin classes in *in vivo* 'protection' (Table 7) and their activities in the established mechanisms by which antibodies can promote bacterial killing (Table 6). That the *in vivo* protective activities were antibacterial was established by removing the bulk of the activity with microgram quantities of 569B-LPS (Table 7). We should have been surprised had complement-dependent activities been effective in the lumen of the intestine. Intraluminal secretory

IgA was very efficient at 'protection' and we wonder whether it might be even more efficient when secreted through the intestinal epithelium. Although some of the differences in *in vivo* protective activities per unit mass of anti-569B-LPS antibody are clearly significant (e.g. IgM *versus* IgG), the range of activities covered by the different immunoglobulin classes was far less than in the vibriocidal and opsonic assays. On a molar basis, anti-569B-LPS IgA, IgM or IgG antibodies had very similar protective activities, in the ratio 6:5:7. Expressed on a molar rather than weight basis the dominance of IgM activity in the vibriocidal and opsonic assays is even greater than illustrated in Table 6.

Fubara and Freter (1973) reported that IgA antibodies to *Vibrio cholerae* which lacked the ability to kill *in vitro* in the presence of complement, would 'protect' adult mice subjected to infection with *Vibrio cholerae* in ligated small bowel loops. We believe that the present paper and that of Steele *et al.* (1974), represent the first reports of antibacterial immunity mediated *in vivo* in intact animals by secretory IgA antibodies. Our observation that dog anti-569B-LPS antibodies of the IgA, IgM and IgG classes have similar protective abilities *in vivo* substantiates the findings of Steele *et al.* (1974), and suggests that there may be an antibacterial mechanism in the intestine common to all three classes.

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