The Spontaneous Release of Somatic Antigen from *Vibrio cholerae*

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SUMMARY

Culture supernatants of Ogawa and Inaba strains of *Vibrio cholerae* contained somatic antigen, judging by their ability to inhibit the vibriocidal action of homologous antisera. Antigen was readily detectable during the exponential phase of growth. The release of antigen was not entirely dependent on cell lysis or death, nor was the decrease in the pH of the medium during the early phase of growth responsible for antigen release. As the concentration of antigen increased, protein, carbohydrate and 260 nm-absorbing material increased in dialysed supernatants but an intracellular enzyme, malate dehydrogenase, was not detectable up to 40 h growth. The antigen demonstrable in supernatants at 40 h accounted for more than a third of the total capacity of the cultures to inhibit vibriocidal antibody. Ethylenediaminetetra-acetate not only increased the release of antigen in suspensions of agar-grown vibrios but also released intracellular substances. Supernatants of Ogawa cultures were distinctly more inhibitory than Inaba supernatants but both were equally effective in gel diffusion precipitation.

INTRODUCTION

It has been known for many years that significant amounts of endotoxin, somatic antigen or immunogen may be found in simple filtrates or supernatants of cultures of Gram-negative enteric bacilli (Milner, Rudbach & Ribi, 1971). Several investigators have demonstrated somatic antigen in the bacteria-free fluids of broth cultures or suspensions of agar-grown *Vibrio cholerae*. Gallut (1962) obtained the antigen by incubating agar-grown organisms in 0.5 % glucose and considered the resulting fall in pH to be important in releasing wall antigen. Kaur & Shrivastav (1964) prepared antigenic polysaccharides from the supernatants of centrifuged broth cultures. Finkelstein, Norris & Dutta (1964) found chick embryo toxicity and antigen neutralizing the vibriocidal activity of antisera in filtrates of 12 to 36 h broth cultures. The mouse-protective lipopolysaccharide of Watanabe & Verwey (1965) was prepared from the broth culture supernatants of an Ogawa strain of *V. cholerae*, but an Inaba strain did not readily liberate the mouse-protective antigen (Verwey, Watanabe, Phillips & Williams, 1965). Holmgren, Lönnroth & Ouchterlony (1971) demonstrated somatic antigen in broth culture filtrates of both Inaba and Ogawa types by double diffusion and separated this antigen from other precipitinogens by filtration through agarose.

In our earlier work (unpublished) significant amounts of somatic antigen were found in the boiled supernatants of suspensions of agar-grown *Vibrio cholerae*. However, it was not possible to determine whether the antigenic material was liberated by autolysis or was released from living organisms. The use of a diffusible fluid medium appeared to provide a more effective means of studying the release of antigen in relation to growth and death of

vibrios. Consequently, supernatant fluids of broth cultures of Ogawa and Inaba strains have been examined for their ability to neutralize vibriocidal antisera and for their content of heat-stable precipitating antigen at successive stages of growth relative to other bacterial constituents and the pH of the culture. The use of vibriocidal antibody inhibition as an indicator of somatic antigen seemed justified by the report of Neoh & Rowley (1970) that boiled vibrios completely absorbed vibriocidal antibody from anti-live vibrio serum and by the observation that heating did not destroy the ability of purified antigen to remove the vibriocidal antibody from antisera to whole vibrios (Watanabe & Verwey, 1965; Verwey *et al.* 1965).

METHODS

Bacterial strains. Ogawa strains of *Vibrio cholerae*, NIH41, VC12, 17, IDH53 and 3083-2, and Inaba strains, NIH35A₃, 569B and BRL7738, were obtained from Dr R. A. Finkelstein. Strains NIH41(V) and v86 were supplied by Dr W. F. Verwey; NIH41 and NIH41(V) were originally from the same source but had been maintained in different laboratories for more than ten years. Cultures were preserved in lyophilized form and were checked frequently for smoothness and failure to agglutinate in anti-rough strain serum.

Culture media. Fluid cultures were made in Trypticase Soy Broth (BBL, Cockeysville, Maryland, U.S.A.) and in a semi-synthetic broth (Syncase; Finkelstein, Atthasampunna, Chulasamaya & Charunmethee, 1966). This medium contained 99.5 % (w/w) of diffusible solids. Trypticase Soy Agar (BBL), in 100 cm diam plates, was used for colony counts and for bacterial suspensions. About 30 mg (dry wt) of bacteria/plate were obtained from confluent growth.

Growth in Syncase broth. Syncase broth (100 ml/500 ml flask) was inoculated with an 8 h soy broth culture diluted to provide a starting concentration of 1000 viable organisms/ml in the 100 ml of medium. Flasks were incubated at 36 to 37 °C in a water-bath shaker at 100 oscillations/min. At intervals 1 ml samples were removed for colony counts. At the same time a 10 ml sample was removed to which was added 0.1 ml of 1 % Thimerosal (Eli Lilly and Co., Indianapolis, Indiana, U.S.A.). A portion of this sample was diluted 1 in 2 for extinction measurement at 520 nm (Spectronic 20, Bausch and Lomb, Rochester, New York, U.S.A.); an extinction value of 3 represented about 2 mg dry wt bacteria/ml. After pH determination, the remainder was centrifuged at 12000 g for 30 min at 4 °C. The supernatant fluid was dialysed against three changes of distilled water (at least 50 vol. each) and analysed serologically and chemically.

Chemical analysis. Supernatant extinction at 260 nm relative to that at 280 nm (DU Spectrophotometer, Beckman, Fullerton, California, U.S.A.) was taken as an indication of nucleic acid content. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Carbohydrate as glucose was measured by the phenol-sulphuric acid reaction (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Malate dehydrogenase was determined with reagents obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A. (Sigma Technical Bulletin no. 340-UV, 1966). One unit of enzyme activity was defined as that amount required to cause a ΔE_{340} of 0.001/min of a mixture containing 0.2 mg NADH and 0.1 mg oxaloacetic acid in 3 ml of 0.1 M-phosphate buffer (pH 7.5) in a 1 cm cell.

Inhibition of vibriocidal antisera. The highest dilution of antiserum killing 50 % of the test organisms (50 % vibriocidal dose: VD₅₀) was determined as described by Pike & Chandler (1969). The specific inhibition of vibriocidal antibody by antigen preparations was measured as the final dilution of antigen required to cause 50 % inhibition of 10 VD₅₀ (Finkelstein, 1962). The 50 % end-point of vibriocidal antibody inhibition (VAI₅₀) was

Fig. 1. Capacity of culture supernatants of strains of *Vibrio cholerae* to inhibit vibriocidal antibody in relation to the colony count, culture density, pH and age of the culture. ●, Colonies/ml; ○, pH; ▲, E_{520} ; —, Ogawa NIH41; - - - -, Inaba NIH35A₃; solid bars, NIH41 VAI₅₀; cross-hatched bars, NIH35A₃ VAI₅₀.

estimated by plotting the survival percentages of the test inoculum against the volumes of inhibitor on log-probit paper (Pike & Chandler, 1969).

Precipitin reaction. Gel double diffusion was performed in 5 cm plastic Petri dishes using wells 5 mm in diam, 4 mm apart, in a gel consisting of 0.8 % agarose (Bausch and Lomb) and 1 % sodium azide. The gel layer was 2 mm thick. After 2 days at room temperature (23 to 25 °C), the titre was recorded as the reciprocal of the highest dilution of antigen causing a deviation of a reference antigen-antibody precipitin band (Nilsson, 1968).

RESULTS

Strains NIH41 and NIH35A₃ were grown in Syncase broth and observed over a period of 64 h (Fig. 1). At 8 h, when the cultures were in the early exponential phase of growth, the VAI₅₀ of the NIH41 culture had increased from 0 to 100. At 12 h, near the end of the exponential phase, the VAI₅₀ had increased to 5000. From this point until 64 h the VAI₅₀

Table 1. *Inhibition of vibriocidal antibody and precipitating antigen titre of supernatants in relation to viability and culture density of Vibrio cholerae broth cultures at 15 and 40 h*

Type	Strain	Colonies/ ml ($\times 10^{-9}$)		E_{520}		VAI ₅₀ *		Precipitating antigen titre†	
		15 h	40 h	15 h	40 h	15 h	40 h	15 h	40 h
Ogawa	NIH41	14.8	1.3	3.04	2.74	10000	40000	2	8
	NIH41(v)	12.9	1.8	2.52	2.80	8600	14000	4	8
	VC12	15.8	3.3	2.80	2.74	6250	15000	2	8
	17‡	14.5	2.3	2.32	2.80	3300	7700	4	8
	IDH53	13.5	2.2	2.92	3.06	14700	50000	2	8
	3083-2‡	4.4	1.0	2.20	4.38	23000	27000	2	8
	Average	12.7	2.0	2.63	3.09	10975	25617	—	—
Inaba	NIH35A ₃	12.9	0.9	2.64	2.92	30	160	4	8
	569B	14.0	0.02	2.56	2.80	30	180	2	4
	v86‡	7.8	0.5	2.96	2.60	190	780	4	8
	BRL7738	7.8	0.6	2.38	2.56	40	110	4	8
	Average	10.6	0.26	2.63	2.72	73	308	—	—

* Reciprocal of dilution of supernatant required for 50% inhibition of 10–50% vibriocidal doses of homologous immune serum tested on homologous organisms.

† Reciprocal of highest dilution of heated (100 °C, 30 min) supernatant showing reaction in gel diffusion test.

‡ El Tor biotype.

continued to increase but at a much slower rate. This rate of increase in VAI₅₀ from 12 to 64 h was not proportional to either the fall in colony count or to the change in culture density. The culture of strain NIH35A₃ followed essentially the same course as NIH41 with respect to viability, density and pH, but the capacity to inhibit vibriocidal activity of serum at all stages of growth was about 100- to 1000-fold less. However, measureable amounts of inhibiting activity were present at 8 and at 12 h.

The marked difference between NIH41 and NIH35A₃ in liberation of inhibitory antigen suggested the examination of other strains of Ogawa and Inaba types. The analysis of 15 and 40 h Syncase broth cultures of six Ogawa and four Inaba strains is presented in Tables 1 and 3. Although there was strain variability in the colony counts (Table 1) at 15 h, there was no consistent difference between Ogawa and Inaba strains at this time. The lower counts for all Inaba strains at 40 h, however, suggest that these organisms die more rapidly as cultures age. On the other hand, there is no definite evidence from the extinction values that Inaba strains autolyse more rapidly than Ogawa strains. At both 15 and 40 h the supernatants of Inaba cultures had consistently lower VAI₅₀ titres than Ogawa supernatants which were one hundred or more times higher.

The difference between Ogawa and Inaba VAI₅₀ titres (Table 1) could have been due to the particular antisera used. An Ogawa and an Inaba supernatant were tested against several homologous antisera (Table 2). The observed variation among different antisera was not sufficient to account for the difference between Ogawa and Inaba titres.

Culture supernatants were also examined, after heating in boiling water for 30 min, for their ability to react in gel diffusion against homologous immune serum (Table 1). Unexpectedly, the Inaba supernatants showed the same order of reactivity as the Ogawa supernatants.

No characteristic differences between Ogawa and Inaba supernatants with respect to pH, protein, carbohydrate or material absorbing at 260 nm could be demonstrated (Table 3).

Table 2. Capacity of supernatants from 40 h broth cultures to inhibit the vibriocidal antibody in different homologous immune sera

Supernatant	Rabbit	Immunized with	VAI ₅₀ *
Ogawa strain NIH41	39	Live NIH41	35 000
	39	Live NIH41	40 000
	46	Live NIH41	4 000
	C1	Live 17	2 100
	C2	Phenol-killed NIH41	5 900
Inaba strain NIH35A ₃	42	Live NIH35A ₃	160
	34	v86 antigen†	160
	55	Live v86	110
	X	Live 569B‡	800

* Reciprocal of dilution of supernatant required for 50 % inhibition of 10 50 % vibriocidal doses of immune serum tested on organisms of strain NIH41 (Ogawa supernatants) or strain NIH35A₃ (Inaba supernatants).

† Mouse protective antigen (Verwey *et al.* 1965) provided by Dr W. F. Verwey.

‡ Antiserum provided by Dr R. A. Finkelstein.

Table 3. Analysis of supernatants of *Vibrio cholerae* broth cultures

Type	Strain	pH		Protein (μ g/ml)		Carbohydrate (μ g glucose/ ml)		E_{260} E_{280}	
		15 h	40 h	15 h	40 h	15 h	40 h	15 h	40 h
Ogawa	NIH41	6.9	8.7	5	83	16	60	0.05/0.0	1.57/0.83
	HIN41(V)	6.5	8.4	7	80	9	41	0.18/0.08	1.30/0.75
	VC12	7.6	8.1	5	52	21	35	0.16/0.08	0.90/0.53
	17*	7.3	8.5	20	45	15	39	0.32/0.15	1.00/0.62
	IDH53	6.6	8.3	5	50	8	39	0.06/0.0	0.42/0.25
	3083-2*	6.6	7.8	17	32	11	20	0.03/0.0	0.30/0.20
	Average	6.9	8.3	10	60	13	39	0.13/0.05	0.92/0.53
Inaba	NIH35A ₃	6.8	8.5	18	47	19	54	0.35/0.16	0.87/0.45
	569B	6.6	8.5	13	118	22	59	0.20/0.07	1.27/0.77
	v86*	7.3	8.6	17	57	14	38	0.18/0.04	0.32/0.18
	BRL7738	6.7	8.3	8	29	13	16	0.04/0.0	0.15/0.08
	Average	6.9	8.3	14	63	17	42	0.19/0.08	0.65/0.37

pH values were measured before and protein, carbohydrate and extinction measurements were made after dialysis of supernatants.

* El Tor biotype.

After an initial drop to around pH 6 (Fig. 1), the pH of all cultures increased to between 7.6 and 8.7 by 40 h. The increases in protein, carbohydrate, and 260 nm-absorbing material were all roughly parallel to each other and to the increase in the VAI₅₀ titre.

Since a relatively large proportion of the inhibitory antigen was released during the phase of growth when the pH fell below 7 (9 to 16 h), the effect of pH on antigen release was investigated. Organisms (strain NIH41) after 18 h growth on plates were suspended in 0.85 % w/v sodium chloride solution, divided into four equal portions and sedimented by centrifuging. The supernatant fluids were discarded and the pellets suspended in 0.067 M-phosphate buffered saline at pH values of 5 to 8 respectively at concentrations equivalent to about 6 mg bacterial dry wt/ml. After incubation at 37 °C for 6 h, Thimerosal (1:10000) was added and the bacteria were again sedimented. These supernatant fluids were dialysed

Table 4. *Effect of pH on the liberation of antigen which inhibited vibriocidal antibody*

pH	VAI ₅₀ * of supernatant
5.0	10 000
6.0	8 300
7.0	12 500
8.0	25 000

Supernatants were prepared after incubation of suspensions of strain NIH41 for 6 h at the indicated pH. Bacterial concentration was equivalent to about 6 mg dry wt/ml.

* See Table 1 footnote.

Table 5. *Analysis of dialysed supernatants from suspensions of agar-grown Vibrio cholerae*

Each suspension contained 6 mg bacteria (dry wt) suspended in 10 ml saline.

Strain	Supernatants from:	Protein (µg/ml)	E ₂₆₀ /E ₂₈₀	Malate dehydrogenase activity (units/ml)	VAI ₅₀ *	Precipitating antigen titre*
Ogawa, NIH41	Original suspension in saline	29	0.01/0.01	none	640	1
	Sedimented organisms re-suspended in saline	88	0.25/0.17	50	1850	2
	Sedimented organisms re-suspended in 0.12 M-tris (pH 8)	256	0.42/0.39	300	1460	2
	Sedimented organisms re-suspended in 0.12 M-tris (pH 8) containing 0.01 M-EDTA	798	1.26/1.20	2900	4100	4
Inaba, NIH35A ₃	Original suspension in saline	29	0.15/0.11	none	54	2
	Sedimented organisms re-suspended in saline	67	0.47/0.30	38	150	4
	Sedimented organisms re-suspended in 0.12 M-tris (pH 8)	95	0.57/0.37	33	109	4
	Sedimented organisms re-suspended in 0.12 M-tris (pH 8) containing 0.01 M-EDTA	624	2.48/1.71	1490	186	8

* See Table 1 footnote.

against distilled water and tested for inhibition of vibriocidal activity (Table 4). No more antigen was released at pH 5 than at pH 7. The pH 8 supernatant, however, had at least twice as much inhibitory activity as any of the less alkaline extracts.

To examine further the possibility that bacterial lysis accompanied the release of somatic antigen, 15 and 40 h supernatants from Ogawa and Inaba cultures were tested before and after dialysis for malate dehydrogenase. No enzyme could be detected although enzymic activity was readily demonstrable in the supernatant fluid from a suspension of vibrios that had been passed through a French pressure cell.

Ethylenediaminetetra-acetate (EDTA) releases large amounts of lipopolysaccharide from *Escherichia coli* (Leive, Shovlin & Mergenhagen, 1968). The extent to which EDTA enhances the release of vibriocidal-inhibiting antigen from *Vibrio cholerae* was therefore examined. Organisms from 18 h growth on agar plates were suspended in saline and divided into three equal portions. After centrifuging at 12 000 *g* for 30 min, the supernatant

Table 6. *Distribution of antigen which inhibited vibriocidal antibody among fractions of 40 h broth cultures of Vibrio cholerae*

Strain	Fraction	Percentage of total VAI ₅₀ * units
Ogawa, NIH41	Culture supernatant	37
	Disintegrated organisms	
	Supernatant	59
	Sediment	4
Inaba, NIH35A ₃	Culture supernatant	46
	Disintegrated organisms	
	Supernatant	51
	Sediment	3

Organisms were disintegrated by passage through a French press.

* See Table 1 footnote.

fluids were removed and the sedimented bacteria suspended respectively in saline, 0.12 M-tris-HCl (pH 8) and in the same buffer containing 0.01 M-EDTA. Vigorous and repeated agitation on a Vortex mixer (Scientific Industries Inc., Springfield, Massachusetts, U.S.A.) was required to suspend the sticky sediments. After 60 min at room temperature, including the time required for complete dispersal of the packed organisms, the suspensions were again centrifuged. The supernatant fluids, after the addition of Thimerosal (1:10000) along with the supernatant from the original suspension, were dialysed against saline. Analysis of the supernatants from cultures of Ogawa NIH41 and Inaba NIH35A₃ showed that both strains released more antigen on resuspension in saline and in tris than was present in the supernatant from the original suspension (Table 5). The Vortex mixing may have contributed to the antigen release. The presence of EDTA increased the antigen content two- to threefold but also caused a very marked increase in protein, malate dehydrogenase, and material absorbing at 260 nm.

The proportion of inhibitory antigen remaining associated with organisms was measured in cultures of strains NIH41 and NIH35A₃ in Syncase broth after 40 h incubation. A portion of each culture was treated with Thimerosal, centrifuged at 12000 g for 30 min, and the organism pellet suspended in saline and passed through a French press at 12 tons/in² pressure. The suspension of disintegrated organisms was centrifuged at 12000 g, the supernatant fluid removed, and the sediment suspended in a volume of saline equal to that of the original suspension. After exhaustive dialysis, the culture supernatant and the supernatant and suspended sediment from the disintegrated organisms were tested for their ability to inhibit homologous vibriocidal activity of antiserum (Table 6). More than a third of the total inhibiting antigen demonstrable by this procedure was released during 40 h growth.

DISCUSSION

During the growth of *Vibrio cholerae* there is undoubtedly some cellular disintegration even during the exponential phase. This must be minimal, however, since malate dehydrogenase could not be detected even at 40 h, at which time there was no decrease in culture density although more than 85 % of the organisms were dead. The ability of culture supernatants to inhibit vibriocidal antibody increased 100-fold from 8 h to the beginning of the stationary phase but increased only about tenfold from this point until 64 h, the time during which the greatest amount of lysis must take place. It is therefore difficult to escape the

conclusion that bacterial lysis is not entirely responsible for the release of somatic antigen from *Vibrio cholerae*.

The release of inhibiting antigen in the presence of EDTA accords with the observations of Leive *et al.* (1968) on *Escherichia coli* and several strains of *Salmonella*, although the conditions of the experiments were not entirely comparable. Repaske (1958) has shown that *Pseudomonas aeruginosa* is more susceptible to lysis by EDTA in the early exponential phase than in the stationary phase. Hence, the effect of EDTA on *Vibrio cholerae* might be more marked if younger organisms were used. Since the maximum effect of EDTA on the bacterial wall is attained within a few minutes (Eagon & Carson, 1965; Leive *et al.* 1968), a shorter exposure than that used in our experiments might release less of the intracellular material.

In spite of the variability in the amount of culture supernatant required to neutralize 10 VD₅₀ of different homologous antisera, the supernatants of Inaba strains were distinctly less inhibitory than those of Ogawa strains. Such a difference would be expected if the Inaba vibriocidal system required more antibody or if Inaba antibody was less avid than the Ogawa. Data supporting either of these possibilities, however, are not available. The difference did not appear to result from the liberation of smaller amounts of antigenic material from Inaba strains, because Inaba and Ogawa supernatants were comparable in precipitation with homologous antisera. These results are relevant to the observation of Verwey *et al.* (1965) that the Inaba mouse-protective antigen was not readily liberated into culture supernatants. These authors also noted that strain v86 produced a larger amount of mouse-protective antigen than strain 35A₃, just as we observed a v86 supernatant to have a greater capacity to neutralize vibriocidal antibody.

The fact that more than a third of the total inhibitory antigen is released into the culture fluid during 40 h growth in a medium containing almost completely diffusible material permits the antigen to be readily concentrated and purified without the use of chemical agents for comparison with somatic antigen extracted by conventional procedures.

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