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Serological Properties of γG and γM Antibodies to the Somatic Antigen of *Vibrio cholerae* During the Course of Immunization of Rabbits

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Direct- and passive-agglutinating, complement-fixing, and bactericidal properties of γG and γM antibodies produced in rabbits inoculated with live *Vibrio cholerae* were determined at intervals over a period of 345 days. Although γM antibody titers increased more rapidly than γG during the initial stages of antibody production, the titers of γG and γM declined proportionally during a 3-month rest period and increased proportionally after a booster injection. The relative titers of γM as determined in the four serological procedures remained fairly constant throughout the period of observation. In contrast, early γG was less effective than late γG in vibriocidal, complement-fixing, and passive-hemagglutinating activity. At no stage of immunization was the agglutinating ability of γG affected by 2-mercaptoethanol, but its complement-dependent activity was markedly reduced, more so in early serum than in late. The heat lability of early γG approached that of γM, but γG became more resistant to heat in later stages of immunization.

Immunity in cholera shows a high degree of correlation with the bactericidal antibody titer (17). Vibriocidal activity of serum is due to antibodies to the heat-stable somatic antigens of *Vibrio cholerae* (8, 20, 33) and resides in both γG and γM fractions (2, 16, 22). On a weight basis, however, γM is considerably more effective than γG (22). Not only do the proportions of γG and γM change during the course of immunization in rabbits but previous observations (22) suggest that the serological activity of γG may show appreciable variation at different stages of antibody formation.

The purpose of these experiments was to compare the serological activity of separated immunoglobulin fractions of early and late immune rabbit sera as measured by the vibriocidal test, the ability to effect both direct and passive agglutination, and the capacity to fix complement.

MATERIALS AND METHODS

Culture. Ogawa strain NIH 41 of *V. cholerae*, originally obtained from R. A. Finkelstein, was used for immunization and for the preparation of antigens for serological tests. Subcultures made from stock lyophilized material were checked for smoothness on BBL Trypticase Soy Agar (TSA).

Preparation of immune sera. Young adult rabbits were injected intravenously with live suspensions made from 24-hr cultures on TSA diluted to the desired concentration of colony-forming units. Five rabbits received identical treatment at the start of the experiment, but only two survived the entire period of observation of 345 days. The schedule of injections and bleedings is given in Table 1. Equal amounts of serum from all surviving rabbits were pooled at each bleeding. Pools 1, 2, and 3 consisted of serum obtained 7 days after one, two, and three weekly injections, respectively. Pool 4 was serum obtained after a rest period of 188 days followed by two more injections. A bleeding 7 days after a final booster injection on day 336 provided serum for pool 6.

Immunoglobulin separation. Serum was mixed with an equal volume of 36% sodium sulfate (13), incubated 1 hr at 37 C, and centrifuged at 12,000 × *g* for 30 min. The supernatant fluid which contained only traces of antibody activity was discarded, and the precipitate was dissolved in 0.01 M phosphate buffer, *p*H 8.6. After dialysis overnight against 100 volumes of the same buffer, the globulins, precipitated from each 12.5 ml of serum, were chromatographed on 5 g of diethylaminoethyl (DEAE) cellulose (Schleicher and Schuell Co., Keene, N.H.) as previously described (22). Fractions I, II, and III (Table 1) were eluted with 0.01, 0.05, and 0.3 M phosphate buffers, respectively. Fraction I was considered to be γG and fraction III was γM immunoglobulin.

Bacterial agglutination. For bacterial agglutination (BA), titers for live organisms were determined as described previously (22).

Passive hemagglutination migration. The capillary method of Severson and Thompson (29), adapted to passive hemagglutination by Johnson et al. (12), was followed in general. Sheep cells (Colorado Serum Company) obtained in Alsever's solution were washed three times and suspended in 2.5% concentration in hemagglutination (HA) buffer (Difco). A volume of this cell suspension was mixed with an equal volume of *V. cholerae* Ogawa somatic antigen in the desired concentration. After incubation at 37 C for 30 min, the cells were washed twice with HA buffer and resuspended in the same buffer to give a concentration of 1%. Somatic antigen was prepared by the method of Gallut (9). Before using the antigen to coat cells, the solution was made 0.02 N with sodium hydroxide, heated in a boiling water bath for 5 min, and neutralized with hydrochloric acid. The concentration of antigen used was the lowest concentration found by a box titration to give maximum titer with anti-Ogawa serum. In the system used, this concentration was 400 μg/ml. For the hemagglutinin titration, double dilutions of the fraction to be tested were individually prepared in HA buffer containing 0.2% bovine serum albumin and 0.002% Tween 80. To 0.2 ml of each dilution, an equal volume of 1% coated-cell suspension was added, mixed, and allowed to stand at room temperature for 30 min. Micro hematocrit capillary tubes (1.5 by 75 mm) were filled in triplicate from each fraction-cell mixture, flame sealed at the dry end, and centrifuged 1 min in an IEC model MB Micro capillary centrifuge. The tubes were attached to a Plexiglas plate fixed at a 45-degree angle and placed at 4 C. When the cells in the negative control had migrated approximately 30 mm, the plates were placed horizontally and the length of the streamers was measured. The 50% passive hemagglutination migration (PHM_{50}) was calculated by interpolating between the dilutions bracketing the 50% end point as described by Johnson et al. (12). The coefficient of variation for replicate determinations was about 12, provided the same lot of sheep blood was used. Since it was found that determinations made with different lots of sheep blood might vary several hundred per cent, it was necessary to check each new lot of blood against a standard serum. Only those lots of blood which gave comparable results with this serum were used to obtain the titers reported.

Vibriocidal antibody titration. The method of Finkelstein (8), modified as previously described (22), was used to determine the dilution killing 50% of the inoculum (VD_{50}).

Complement fixation. For complement fixation (CF), the macromethod described by Lennette (14) was used, taking as the end point the highest dilution showing at least 5% fixation. The antigen was the same as that used for passive hemagglutination except that it was not treated with sodium hydroxide. Such treatment was found to interfere markedly with the ability of the antigen to fix complement. Anticomplementary fractions were pretreated with complement as described by Lennette (15).

Reduction and alkylation. Equal volumes of serum fraction and freshly prepared 0.2 M 2-mercaptoethanol (ME) in HA buffer were mixed and left at room temperature for 2 hr. A weighed amount of iodoacetamide (IOD), sufficient to make the solution 0.02 M, was then added and the mixture was refrigerated overnight at 4 C. The preparation was then dialyzed against HA buffer at 4 C for 24 hr and changed to fresh buffer after 6 to 8 hr. Since the bacteriostatic effect of residual ME might lead to erroneous results in the vibriocidal test, it was shown that these concentrations of ME and IOD after equivalent dialysis were not vibriocidal. Portions of fractions to be used in serological determinations without reduction were treated in the same manner, substituting HA buffer for the ME solution.

RESULTS

The antibody response of rabbits as measured by BA on serum pools and the chromatographic distribution of the agglutinins is shown in Table 1. Nearly maximal titer was attained after the

TABLE 1. *Immunization of rabbits and chromatographic distribution of agglutinins in pools of serum*

Day	Live vibrios injected	No. of rabbits	Serum pools			Per cent distribution of agglutinins in chromatographic fractions[a]		
			Designation	Amt (ml)	Titer[b]	I	II	III
0	2×10^8	5						
7	8×10^8	5	Pool 1	86	1,280	2	0	98
14	1.6×10^9	3	Pool 2	48	5,120	2	<1	98
21		3	Pool 3	24	2,560	6	<1	94
202	1.6×10^9	3						
210	3×10^9	3						
217		3	Pool 4	24	5,120	22	1	77
304		2	Pool 5	48	320	34	5	61
336	3×10^9	2						
345		2	Pool 6	24	10,240	39	1	60

[a] Amount of agglutinin in each of the three serum fractions was represented by the product of the titer and the volume of fraction.

[b] Bacterial agglutination.

second injection, at which time 98% of the agglutinin activity was present in the γM fraction (fraction III). A significant proportion of γG (fraction I) agglutinin did not appear until after the fifth injection (pool 4), when 22% of the activity was in fraction I. In the absence of antigenic stimulation from day 217 to day 304, there was a 16-fold drop in titer with only a slight relative increase in the γG. An additional injection on day 336 resulted in a prompt restoration of the agglutinin titer without a significant change in the proportion of γG and γM.

For γG and γM fractions of each serum pool, the BA titer, PHM_{50}, VD_{50}, and CF titer were determined. The titers, expressed as a dilution of the original volume of serum, are graphically presented in Figures 1 and 2. Although γG (Fig. 1) was readily detectable in pool 1 by BA and VD_{50} titrations, the PHM_{50} and CF titers were less than 1 and were measurable only because the antibody was more concentrated in the fractions than in the original serum pool. The CF titer of pool 2 was also less than 1. The titers by all four serological procedures were generally parallel except for the relatively low PHM_{50} and VD_{50} titers of γG in the early sera, pools 1, 2, and 3. This deficiency is further documented in Table 2, which shows that the ratio of PHM_{50} to BA in pools 4, 5, and 6 is 12 to 19 times the comparable ratios in pools 1, 2, and 3. The early γG, therefore, is relatively less effective in binding together antigen-coated cells. The early γG fractions were also relatively deficient in their ability to kill *V. cholerae*. This is evident in the VD_{50}-BA ratios indicated in Table 2. These ratios for pools 4, 5, and 6 are from 6 to 27 times the ratios for pools 1, 2, and 3.

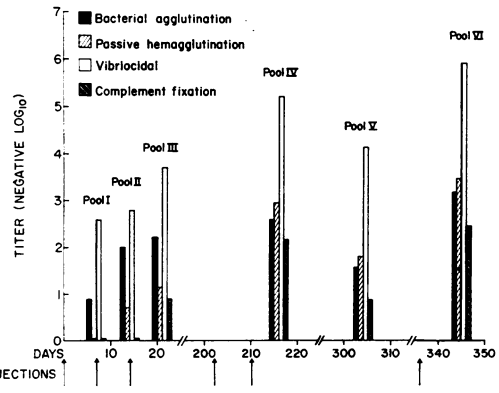

FIG. 1. *Titers of γG fractions in terms of original volume of pooled serum at successive stages of immunization.*

In contrast to γG, the γM titers (Fig. 2) approached the maximum following a single injection. The average percentage decrease in titer during the 87-day interval between pools 4 and 5 was practically the same for γM as for γG, about 88%. The PHM_{50}-BA and VD_{50}-BA ratios (Table 2) remained fairly constant throughout the period of observation.

The relative amounts of serological activity contributed by γG and γM in the four tests applied to the six serum pools are shown in Table 3, expressed as the ratios of γM to γG titers. As expected, γM accounted for most of the serological activity of each serum. Even in the later serum pools, γM titers exceeded γG except in CF.

Reduction with ME followed by alkylation with IOD resulted in at least a 99% decrease in

TABLE 2. *Comparison of bacterial agglutinin (BA), passive hemagglutination migration (PHM_{50}), and vibriocidal (VD_{50}) activity of rabbit serum fractions during immunization*

Fraction	Pool	Titer[a]				Ratio		
		BA	PHM_{50}	VD_{50}	CF	PHM_{50}/BA	VD_{50}/BA	BA/CF
γG	1	7.2	0.9	417	0.36	0.13	58	20
	2	32	4.4	644	0.8	0.14	20	40
	3	86	14	5,360	8.6	0.16	62	10
	4	374	912	166,300	150	2.44	445	2.5
	5	37	68	13,160	7.0	1.84	356	5.3
	6	1,490	3,040	803,900	298	2.04	539	5.0
γM	1	442	2,120	1,228,000	44	4.80	2,779	10
	2	1,410	9,140	3,341,000	70	6.49	2,373	21
	3	1,330	6,350	2,933,000	67	4.77	2,204	20
	4	1,330	4,740	2,701,000	67	3.56	2,029	20
	5	67	573	323,000	3.4	8.60	4,820	20
	6	2,510	26,100	5,155,000	63	10.40	2,055	40

[a] Adjusted to original volume of serum. CF, complement fixation.

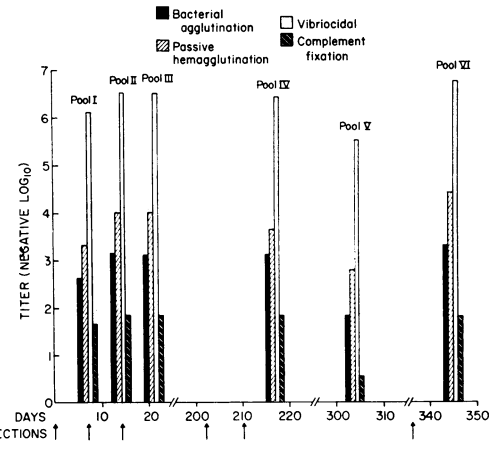

FIG. 2. *Titers of γM fractions in terms of original volume of pooled serum at successive stages of immunization.*

TABLE 3. *Ratios of γM to γG titers[a] by four serological procedures during the course of immunization[b]*

Serum pool	BA	PHM_{50}	VD_{50}	CF
1	61	2,359	2,945	122
2	44	2,078	5,187	88
3	15	454	547	7.8
4	3.6	5.2	15	0.5
5	1.8	8.4	25	0.5
6	1.7	8.6	6.4	0.2

[a] Adjusted to the original volume of serum.

[b] Abbreviations: BA, bacterial agglutination; PHM_{50}, 50% passive hemagglutination migration; VD_{50}, 50% vibriocidal dose; CF, complement fixation.

all titers of γM fractions. Treatment with ME and IOD also partially destroyed some of the serological activities of γG (Fig. 3), and its effect on γG in early serum pools was more marked than on γG produced in the later periods of antibody formation. Although the capacity of γG to agglutinate bacterial cells was completely resistant, only 5% of the vibriocidal activity of pool 1 γG remained after treatment. The ME resistance of vibriocidal capacity increased progressively to 59% in pool 4. The small amount of CF activity present in pools 1 and 2 before treatment was no longer detectable after reduction. At least 75% of the CF activity of γG in later serum samples was destroyed.

Since it has been shown that γG is more resistant to heat than γM (24), it was of interest to compare the heat stability of γG produced at different stages of immunization. Samples of fractions were diluted with equal volumes of HA buffer and suspended in a water bath maintained within 0.5 C of the desired temperature. The effect of heat is shown in Fig. 4. The vibriocidal activity of γM (pools 4 and 5) was reduced at 60 C and was almost completely destroyed at 65 C. Late γG (pool 4), in contrast, was not affected at 65 C. The activity of the earliest γG (pool 1), however, was 80% destroyed at 65 C. Pool 2 γG showed increased resistance to heat as compared to pool 1.

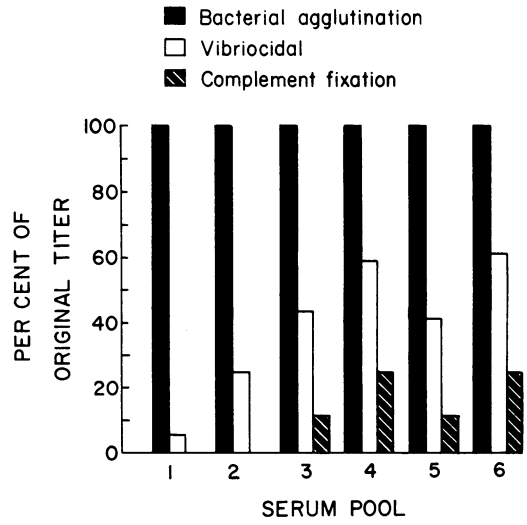

FIG. 3. *Effect of 2-mercaptoethanol on complement fixing, vibriocidal, and bacterial agglutination titers of γG fractions at successive stages of immunization.*

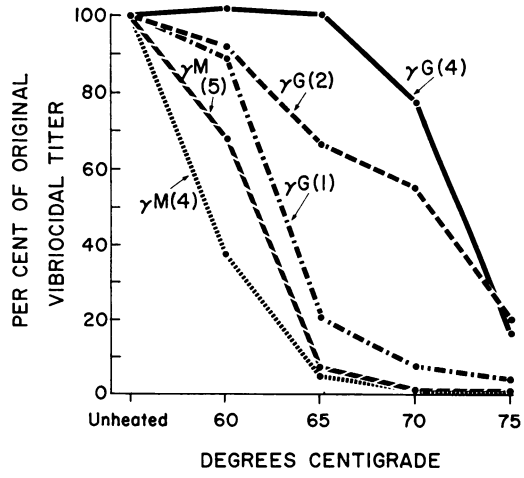

FIG. 4. *Effect of heat on vibriocidal activity of γG and γM fractions. Numbers in parentheses refer to serum pools. Samples were heated for 30 min at indicated temperatures.*

TABLE 4. *Effect of treatment with complement and with mercaptoethanol on complement-fixing titer of γM fraction*

Treated with	Antigen present	Titer
Untreated	+	64
	−	16
Complement	+	64
	−	4
Mercaptoethanol	+	4
	−	4
Complement and mercaptoethanol	+	<4
	−	<4

In attempting to determine the specific CF capacity of serum fractions, it was found that all γM preparations were anticomplementary (AC) up to dilutions of 1:16 or 1:32. In contrast, no γG fraction fixed complement in the absence of antigen at a 1:2 dilution. ME has been reported to reduce the AC activity of whole guinea pig serum (1) but ME would also affect the specific activity of γM. By pretreating γM fractions with complement by the method of Taran (31) as described by Lennette (15), it was possible to determine specific CF titers. The effect of treating one γM fraction with complement and ME is shown in Table 4. Both complement and ME greatly reduced the capacity of γM to fix complement in the absence of antigen. Treatment with complement did not reduce the specific titer, whereas ME did.

DISCUSSION

Although the somatic antigens of gram-negative bacilli elicit an immune response which appears to be predominantly γM, a considerable proportion of the antibody activity may be found in the γG fraction of serum (7, 22, 23, 26). The apparent distribution of antibody activity among the immunoglobulins depends to a large extent on the serological procedure used (21, 25). Thus, in the present study, the greater portion of the activity of late sera (pools 4, 5, and 6) as measured by CF appeared to be in the γG fractions, although the agglutinating and vibriocidal activities were greater in the γM fractions (Fig. 1 and 2).

Not only was the decline in γG and γM titers about equal during the 88 days between pools 4 and 5 but also the rises in γG and γM titers after the last injection were similar. This response of rabbits to intravenous injection of live *V. cholerae* is qualitatively similar to that of cholera patients as reported by Merritt and Sack (16) and by Ahmed et al. (2). Both groups of investigators interpreted the simultaneous rise in ME-resistant and ME-sensitive antibody, observed in most cholera patients, as a secondary response occurring in individuals who had prior exposure to *V. cholerae*. The continued synthesis of γM antibody to lipopolysaccharide antigens in both man and the rabbit seems to differ from the response to some other antigens (10, 32) in which γM can no longer be detected in the serum when the level of γG reaches its maximum.

The observation that the ratio of vibriocidal to agglutinin titer of γG fractions at different stages of immunization was more variable than the ratio of these titers of γM fractions (22) has been confirmed and correlated with the relative efficiency of these immunoglobulins to fix complement and to prevent the migration of erythrocytes coated with somatic antigen. There was no significant change in γG with respect to the ratio of PHM_{50}, VD_{50}, and CF titer to BA titer through the first three bleedings, although the amount of γG, measured by BA, increased more than 10-fold during this time. A marked change in these ratios was evident, however, when the rabbits were again inoculated after a prolonged rest. Once the change had occurred, the ratios remained relatively constant even when the titers declined in the absence of further antigenic stimulation.

The poor correlation between BA and PHM titers of γG fractions was an unexpected result. PHM titers, relative to BA titers, were low in early γG and increased more than 10-fold in late sera. In contrast, the γM titers by these two procedures were fairly parallel during the whole period of observation. The weak PHM activity of early γG may be related to the weak affinity of early γG (21). The less avid antibody may be less capable of holding antigen-coated erythrocytes together, thereby permitting them to migrate in the capillary test. The same lack of binding power may not be evident in bacterial agglutination where a positive result is determined by different criteria.

A more important finding, revealed by the examination of sera at different stages of antibody formation, was the increase in resistance of γG to reduction by ME. This difference is of particular interest in view of the conflicting reports of other investigators. ME has been reported to destroy or reduce CF activity of γG from human (11, 19, 34), rabbit (4, 11, 19, 30, 35), mouse (1), and sheep serum (30). Others have found that reduction had no effect on CF activity of human (3, 18, 28) and guinea pig (6) γG. The ability of mouse (1) and rabbit (27) γG to lyse sheep

erythrocytes in the presence of complement was destroyed by ME, but ME had no effect on the bactericidal property of rabbit γG for *Salmonella typhimurium* (26). It is evident that under certain conditions reduction can interfere with the CF capacity of γG. Brinkerhoff and Rose (4) have suggested that the antigen is an important factor in determining the ME-sensitivity of γG. Our observations suggest that another variable involved may be the time at which γG is formed during the immune response. The possibility that the decreased activity of γG fractions after ME treatment was due to contamination with γM was ruled out by the fact that ME had no effect on the BA titer.

Not only was early γG found to be more susceptible to ME than γG produced later but it was also more susceptible to inactivation by heat. Although it is unlikely that resistance to heat and to ME are due to the same molecular characteristics, these effects reflect a relative instability of the early γG molecule. Natural human γG reacting with gonococcal antigens was found to be inactivated by heat more readily than immune γG (5).

A possible explanation for the observed changes in the reactivity of γG might be the appearance in late sera of antibodies to minor antigenic determinants. If this were the case, however, one would expect similar changes in γM. Furthermore, it is unlikely that a broadening of the specificity of antibody could account for the increased resistance to inactivation by heat and ME.

Although cholera in man is an infection confined to the intestinal tract, the correlation observed between vibriocidal titer and resistance (17) and the fact that parenteral administration of vaccine provides some protection against infection suggest that humoral antibody plays some part in immunity. The early appearance of γM vibriocidal activity, the weaker bactericidal capacity of γG, particularly that formed first after antigenic stimulation, and the greater complement requirement of γG (22) indicate that γM antibodies may play an important role in the destruction of *V. cholerae*. It is also suggested that any γG produced in the primary response of human subjects as a result of cholera vaccination may be relatively ineffective vibriocidal antibody as compared to that produced in persons previously exposed to *V. cholerae* antigens.

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