ANTIBACTERIAL IMMUNITY IN EXPERIMENTAL CHOLERA *

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IN an earlier report, we proposed a new approach to the study of immunity in Cholera which would differentiate between antibacterial and antitoxic mechanisms[1]. This depended on the use of 'hybrid' vibrio strains, obtained by genetic techniques, which possessed somatic antigens different from that of *Vibrio cholerae* from which they were isolated and which did not cross react in serological and mouse protection tests[2,3]. In other respects, the hybrids retained the characters of the corresponding parent strains such as the ability to produce the choleragenic toxin (enterotoxin) *in vitro*, haemolytic activity, polymyxin sensitivity/resistance pattern, and nutritional requirements[3]. This was consistent with the theory that the hybrids arose by genetic recombination in the chromosomal region coding for the synthesis of somatic antigens. At present, four vibrio strains are available in such alternative antigenic forms, the parent strains conforming to the somatic antigenic structure of *V. cholerae* (0 group 1)[4] while the hybrids possess a different specificity designated 0–165. By a judicious selection of immunizing preparations and challenge strains for use in experimental animal models, antibacterial and antitoxic immunity patterns should be easily identified and the preliminary results of such a study in the rabbit ileal loop model[5] are presented here.

MATERIALS AND METHODS

Bacterial strains.—*Vibrio cholerae*, Inaba 569 B, and the 0-165 hybrid derived from it, 569 B : SR-165, were used to obtain crude enterotoxin for the preparation of formalinised toxoid and for sensitisation of sheep RBC. For immunization of rabbits, a 0-165 hybrid strain (T 50 : SR-165) and an attenuated strain of *V. cholerae* (C 14-S 5)[6] were employed. Three strains were used for ileal loop challenge experiments. They were T 50 : SR (*V. cholerae*, Ogawa, eltor biotype), T 50 : SR-M1 (an Inaba mutant of T 50 : SR) and T 50 : SR-165 (0-165 hybrid derived from T 50 : SR).

Cholera Enterotoxin and Formalinised Toxoid.—Crude cholera enterotoxin was prepared by inoculating strain Inaba 569 B (or 569 B : SR-165) in Syncase medium[7] supplemented with hypoxanthine (0·01%) and subsequent incubation in shaken cultures for 16 h. at 28° C. After centrifugation, the supernatant (constituting crude enterotoxin) was stored at 4° C. Conversion to toxoid was achieved by the addition of formaldehyde (0·2%) and incubation at 37° C for 4 days and subsequently at room temperature for 3 days. This was then maintained at 4° C. For estimating potency, crude preparations of enterotoxin were diluted 1 : 10 in Brain Heart Infusion (Difco) and filtered twice through membrane filters (pore size, 0·2 μ). Sterile preparations obtained thus were serially diluted in Brain Heart Infusion and 1 ml of different dilutions was introduced into ligated ileal loops of adult rabbits. The loops were examined after 18 h. and the fluid contained in the loops was measured. Maximal distension with fluid was generally observed up to 1 : 200 dilution. Precise quantitation was not attempted and such preparations were assumed to contain not less than 200 loop units[8]. In the skin of guinea-pigs, these preparations gave rise to perceptible induration when 0·1 ml. was injected intracutaneously up to dilutions of $1 : 10^3$ or $1 : 10^4$.

Immunization.—Three groups of rabbits (Groups B, C and D) were immunized while Group A served as controls. Group B rabbits received two subcutaneous doses of 10^9 and $1{\cdot}7 \times 10^9$ viable cells of a 6 h nutrient broth culture of attenuted *V. cholerae*, C 14-S 5 (Ogawa), contained in 0·5 ml and 1·0 ml respectively. Group C rabbits received two subcutaneous doses of 9×10^8 and $1{\cdot}2 \times 10^9$ viable cells of a 4 h nutrient broth culture of the hybrid strain, T 50 : SR-165, contained in 0·5 ml. Group D rabbits received two subcutaneous doses (1 ml) of formalinised (Syncase) toxoid derived from the hybrid strain, 569 B : SR-165. In all cases, the second dose was administered three weeks after the first. Animals were challenged 11 to 21 days after the second immunization. Blood for

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antibody estimates were obtained 6-7 days after the 2nd dose of vaccine (or toxoid) and in the case of controls, 7-14 days prior to challenge. Four animals were challenged at a time, one drawn from each group.

Challenge Experiments.—Rabbits were starved for 48 h. prior to challenge. Three ligated ileal loops were prepared in each animal, in the manner described by De and Chatterjee[5], for challenge with the three test strains. The challenge was contained in 1 ml (10^6 viable cells) of a 10^{-3} dilution of a 3 h. Brain Heart Infusion culture. The loops were examined 18 h. after challenge and the fluid contained in the loop was measured.

Serum Antibody Estimates.—Vibriocidal titers of sera were estimated by the procedure of Finkelstein[9]. If no vibriocidal activity was detected in dilutions of 1 : 50, the serum was considered to lack this antibody. Antitoxin levels were determined by the haemagglutination technique with toxin sensitised sheep RBC as developed by Hochstein *et al.*[10]. 0·3 ml of serial dilutions of sera was mixed with 0·3 ml of sensitised sheep RBC in Perspex haemagglutination plates. Results were read after 2 h. incubation at 37° C followed by overnight refrigeration at 4° C. For tests with sera from Groups C and D, sheep RBC (plain untreated) were sensitised with crude enterotoxin obtained from *V. cholerae,* Inaba 569 B. For sera from the other groups (A and B), the cells were sensitised with a similar enterotoxin prepared from the hybrid strain, 569 B : SR-165. Although it is known that bacterial antigenic components do not interfere in this assay system, the sensitisation procedure resorted to above was a further safeguard against false positive reactions. These tests included controls with known negative and positive sera, the latter being a rabbit antiserum (HA titer 800) obtained after multiple subcutaneous immunization with a toxoid from 569 B : SR-165. In some tests, a monospecific equine antiserum against cholera enterotoxin[11] was also included as control but the haemagglutination observed (HA titer 6,400) with this serum was less marked than that seen with the rabbit antiserum.

Results

Full details of this study are recorded in Table I, and summarised in Table II. At the start, there were eight rabbits in each group but one animal in Group C had to be destroyed as it lost weight considerably during immunization. In the controls (Group A) it will be seen that all the three challenge strains caused fluid accumulation in ligated ileal loops and strain T 50 : SR appeared to be more virulent in this respect than the other two strains derived from it. Two (Nos. 6 and 8) of the 8 control rabbits gave negative loops when challenged with T 50 : SR-M1 and T 50 : SR-165 respectively. None of the animals in this group had HA (haemagglutinating serum antibody to toxin coated sheep RBC) titers at the level of 20, while 2 of them (Nos. 5 and 8) had vibriocidal antibody in their sera to one or more of the challenge strains.

Immunized rabbits (Groups B, C and D) showed significant differences between them in antibody pattern. All the animals in Group B (immunized with *V. cholerae,* C 14-S 5, Ogawa) developed vibriocidal antibody to the challenge strains of *V. cholerae.* In 6 out of 8 animals, Ogawa titers were higher than Inaba while in the other two they were of the same magnitude. With the exception of one rabbit (No. 10) none of the rabbits in this group had vibriocidal antibody to the hybrid challenge strain. In Group C (immunized with the hybrid strain, T 50 : SR-165) the situation was just the reverse with no vibriocidal antibody towards the strains of *V. cholerae* while a uniform titer of 10^4 was observed with the hybrid challenge strain. Group D animals responded to immunization with cholera toxoid (from hybrid strain 569 B : SR-165) with significant HA titers (ranging from 800 to 3200) and good levels of vibriocidal antibody to the hybrid strain. The latter occurrence was not unexpected considering that the crude toxoid should have contained adequate cellular material for antigenic stimulation.

It is noteworthy that none of the animals immunized with the two live vaccine developed antibody to the toxin. This could be correlated with the finding that neither of these two strains produced toxin *in vitro.* As already reported, strain C 14-S 5 was avirulent in experimental animal models[6]. The response in these animals could be different if *in vitro* toxigenic strains, such as *V. cholerae,* Inaba 569 B, were used as live vaccines. However, it was seen that ileal loop fluids of rabbits infected with *V. cholerae,* T 50 : SR and the hybrid, T 50 : SR-165, contained free enterotoxin. This was demonstrated by injecting these fluids, diluted 1 : 5 in Brain Heart Infusion and sterilised by filtration twice through

TABLE I

Ileal loop challenge experiments in rabbits and serum antibody levels before challenge

Immunization	Rabbit No.	Challenge: *V. cholerae* T 50 : SR Ogawa	Challenge: *V. cholerae* T 50 : SR-M1 Inaba	Challenge: 0-165 *Hybrid* T 50 : SR-165	Serum antibody†: Vibriocidal titer T 50 : SR Ogawa	Serum antibody†: Vibriocidal titer T 50 : SR-M1 Inaba	Serum antibody†: Vibriocidal titer T 50 : SR-165 *Hybrid*	Serum antibody†: HA titer
Group 'A'	1	2·13*	1·57	1·09	0	0	0	0
Nil	2	2·00	1·09	1·33	0	0	0	0
(Controls)	3	2·00	1·82	1·20	0	0	0	0
	4	2·05	1·82	0·82	0	0	0	0
	5	1·87	0·85	0·32	10^2	0	0	0
	6	1·80	0·00	0·88	0	0	0	0
	7	2·18	1·91	1·92	0	0	0	0
	8	1·08	0·36	0·00	0	50	50	0
Group 'B'	9	0·00	0·92	1·62	10^5	10^4	0	0
Live vaccine	10	1·05	0·48	2·00	10^5	10^4	10^2	0
V. cholerae	11	0·06	0·00	0·38	10^4	10^3	0	0
C 14–S 5	12	0·00	0·69	1·42	10^4	10^3	0	0
(Ogawa)	13	0·39	1·05	1·90	10^4	10^3	0	0
	14	0·00	0·09	0·62	10^4	10^3	0	0
	15	0·00	0·11	0·05	10^4	10^4	0	0
	16	0·00	0·00	1·63	10^4	10^4	0	0
Group 'C'	17	2·00	2·32	0·00	0	0	10^4	0
Live vaccine	18	1·44	1·74	1·60	0	0	10^4	0
0-165 *Hybrid*	19	1·83	2·20	0·00	0	0	10^4	0
T 50 : SR-165	20	2·09	2·16	0·00	0	0	10^4	0
	21	2·00	2·20	0·00	0	0	10^4	0
	22	1·75	0·50	0·00	0	0	10^4	0
	23	2·41	2·50	0·00	0	0	10^4	0
Group 'D'	24	1·57	1·12	0·00	N.D.	N.D.	N.D.	N.D.
'Syncase' toxoid	25	1·85	1·45	0·00	0	0	10^4	1600 (0)
from	26	1·70	0·94	0·05	0	0	10^4	1600 (0)
569B : SR-165	27	1·45	1·57	0·00	0	0	10^4	3200 (0)
(0-165 *Hybrid*)	28	1·08	1·53	0·00	0	0	10^5	1600 (0)
	29	1·77	1·22	0·00	0	0	10^5	800 (0)
	30	1·78	1·08	0·00	0	0	10^5	1600 (0)
	31	1·81	1·57	0·00	0	0	10^4	1600 (0)

* Fluid volume (ml)/loop length (cm).

† Titer is the reciprocal of the highest dilution causing positive reaction.

Figures in parentheses represent titers prior to immunization (Group 'D').

0 = less than 50 (vibriocidal titer) and less than 20 (HA titer). N. D. = Not determined.

TABLE II

Analysis of ileal loop challenge experiments in rabbits recorded in Table I

Immunization	No. of rabbits	Challenge strain	Fluid in ileal loop: Range	Fluid in ileal loop: Mean	Fluid in ileal loop: Median	Immunity to challenge
Group 'A'	8	Ogawa	1·08–2·18	1·89	2·00	..
Nil		Inaba	0·00–1·91	1·18	1·33	..
(Controls)		Hybrid	0·00–1·92	0·95	0·98	..
Group 'B'	8	Ogawa	0·00–1·05	0·19	0·00	Significant ($P < 0·01$)
Live vaccine		Inaba	0·00–1·05	0·42	0·30	Significant ($P < 0·05$)
V. cholerae		Hybrid	0·05–2·00	1·20	1·52	nil
C 14-S 5 (Ogawa)						
Group 'C'	7	Ogawa	1·44–2·41	1·93	2·00	nil
Live vaccine		Inaba	0·50–2·50	1·95	2·00	nil
0-165 *Hybrid*		Hybrid	0·00–1·60	0·23	0·00	Significant ($P < 0·01$)
T 50 : SR-165						
Group 'D'	8	Ogawa	1·08–1·85	1·63	1·74	nil
'Syncase' toxoid		Inaba	0·94–1·57	1·31	1·34	nil
from 569B : SR-165		Hybrid	0·00–0·05	0·01	0·00	Significant ($P < 0·01$)
(0-165 *Hybrid*)						

membrane filters (pore size, 0·2 μ), into ligated ileal loops of rabbits. Outpouring of fluid was observed in these loops which received 1 ml of the sterile filtrate. Furthermore, this toxicity was completely neutralised by treatment with an equine monospecific antiserum prepared against purified choleragenoid (a naturally occurring toxoid of *V. cholerae*, Inaba 569 B)[11]. This was good evidence that the enterotoxin produced by these two challenge strains were antigenically identical with that produced by *V. cholerae*, Inaba 569 B. Finkelstein has already reported a similar finding with 5 different strains of *V. cholerae*[12]. Nevertheless, as stated above, live vaccine of the hybrid, T 50 : SR-165, did not induce the formation of antibody to toxin in spite of its *in vivo* toxigenicity as observed in rabbit ileal loops.

The ileal loop challenge experiments are equally instructive. Table II may be scrutinised for an analysis of the experiments. Immunity patterns are reasonably well demarcated. Group B rabbits (immunized with *V. cholerae*, C 14-S 5, Ogawa) were protected against challenge with *V. cholerae* (T 50 : SR, Ogawa) and to a lesser extent against challenge by *V. cholerae* (T 50 : SR-M 1, Inaba). In contrast, there was no immunity to challenge by the hybrid strain. Groups C and D were immune to challenge with the hybrid strain, but not to the strains of *V. cholerae*. As antibody to the toxin was not demonstrable in rabbits of Groups B and C, and vibriocidal activity was observed only to the challenge strains to which they were resistant, the immunity observed could be attributed to antibacterial factors alone.

Group D animals (immunized with toxoid) were not resistant to challenge by *V. cholerae* strains in spite of the high HA titers observed in their sera although there was a slight reduction (in fluid accumulation) in loops challenged with T 50 : SR (Ogawa). This is in contrast to the findings of Finkestein[12], who observed antitoxic immunity in this experimental model after immunization with purified choleragenoid. This was effective against challenge by 5 different strains of *V. cholerae*. These divergent observations are difficult to reconcile at present, unless it is attributed to higher antitoxin levels (HA titers from 640 to 10,240) observed in rabbits immunized with choleragenoid. However, our findings clearly show that antibacterial factors alone can provide effective immunity in this experimental model. Several questions remain unanswered. They relate to differences, if any, in the immunogenic efficacy of live and killed vaccines, the duration of such immunity and the potentiating effect that may be expected by fortifying these vaccines with toxoid. Precise information on these points in this and other experimental models can lead to fruitful studies in man.

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