INFECTION AND IMMUNITY, May 1972, p. 662-667

Vol. 5, No. 5
Printed in U.S.A.

Experimental Studies on Cholera Immunization

II. Evidence for Protective Antitoxic Immunity Mediated by Serum Antibodies as Well as Local Antibodies

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Received for publication 2 November 1971

By use of the ileal loop technique, the resistance to challenge with cholera enterotoxin was compared between unimmunized rabbits and rabbits immunized with toxin or toxoids. It was shown that subcutaneous as well as intraintestinal immunization induced protective immunity, the toxin being a better immunogen than Formalin-induced toxoid and much better than heat-induced toxoid. The relation between protection and serum antitoxin titer was poor, e.g., protection was seen in the absence of demonstrable serum antibodies. However, intravenous administration of antitoxic antiserum conferred some protection, suggesting that local as well as serum-mediated antitoxic immunity is operating in the host defence against cholera.

A cell-free culture filtrate from *Vibrio cholerae*, when administered to the gut, can give rise to the classical features of Asiatic cholera, with transport of fluid and ions from the tissues into the gut lumen. A recently characterized protein exotoxin (10, 12, 16), antigenically identical from strains of different serotypes (12), appears to be responsible for this effect and will in this report be referred to as cholera toxin. Besides its toxic action on the gut level, this type of molecule can act as a dermal capillary permeability factor (PF) and gives, on subcutaneous immunization, rise to high levels of neutralizing antibodies in the blood serum, which seem to be practically exclusively of the immunoglobulin G (IgG) class (J. Holmgren, A.-M. Svennerholm, and Ö. Ouchterlony, *to be published*). Neutralizing serum antibodies also develop after immunization with natural or artificial toxoid (8, 10, 11) as well as after cholera infection (1, 4, 11, 14). The functional role of antitoxic immunity for protection against cholera and the significance of serum antibody in comparison to locally formed intraluminal antibodies are unsolved problems which are presently subject to intense investigations employing various animal models (2, 5, 6, 9). This report presents experiments, employing the rabbit ileal loop model (7), which were designed to investigate the protective efficiency of immunization with toxin and toxoids and to compare the subcutaneous and intraluminal routes of immunization. A further aim was to study whether the tentative protection induced by immunization was mediated by serum antibodies, by locally formed intraintestinal antibodies, or both of these types of antibodies.

MATERIALS AND METHODS

Toxins and toxoids. The toxin used for immunization and referred to as IA was the freeze-dried and redissolved retentate after ultrafiltration of culture filtrate material (lot 4493 G from NIH, Bethesda, Md.) from an Inaba strain. This retentate (4493 G_{Ia} in reference 12) was immunologically characterized in a recent report and contained when prepared about 0.1% toxin. To prepare toxoids this material was treated with 0.2% Formalin for 4 days at 35 C, and another portion was heated at 56 C for 45 min, procedures which destroyed the toxic activity of the material as tested in the intradermal (4) and ileal loop systems (7).

The toxin used for challenge of the ileal loops was the lyophilized culture filtrate (lot 001, NIH, Bethesda, Md.) of an Ogawa strain, which prior to use was dissolved and dialyzed for 16 to 20 hr against phosphate-buffered saline. Details on the immunological characteristics of such material are given in reference 12, which showed that the toxin in this preparation is antigenically identical with the toxin in the material used for immunization. From this reference can also be extracted that a blueing dose (1, 4) of the challenge toxin was 9 μg and that the proportion of pure toxin was about 0.01% (w/w). Further work (J. Holmgren, *unpublished data*) has indicated that the dialysis procedure reduced the toxicity of the culture filtrate about fourfold but not its capacity to bind antitoxin antibodies, i.e., the amount of loop-active toxin in 4 mg of challenge material can be estimated to be about 100 ng.

Immunization schedule. Five groups of rabbits, 6 to 8 weeks old, with three animals in each group, were actively immunized with IA. Another two 3-animal

groups were immunized with the Formalin-treated IA and with the heated IA, respectively. The routes of immunization and the intervals between the injections if repeated, as well as the intervals to challenge after the last injection, are reported in Tables 4 and 5. The local immunization of the gut was performed after laparatomy by injecting the antigen into the ileal lumen about 60 cm above the ileocaecal border (Sacculus robundus). All injections consisted of 12.5 mg of IA or toxoid dissolved in 1 ml of phosphate-buffered saline, since this dose on subcutaneous injection was found to be the optimum to induce formation of neutralizing serum antibody (J. Holmgren et al., *to be published*).

In addition, five animals were passively immunized by intravenous injection of 2 or 8 ml of hyperimmune sera against IA having PF neutralizing titers (*see below*) of 4,000 to 6,000. The serum transfusions were performed 4 hr before challenge and immediately after bleeding of the same blood volumes. Animals given these volumes of preimmunization serum served as controls.

Fifteen unimmunized rabbits, 7 to 11 weeks old, were also included in the study as controls.

Ileal loop challenge. The technique of Burrows and Musteikis (3) was employed with minor modifications. The animals, after 36 to 48 hr of starvation but with no water restrictions, were given 5 mg of acepromazine (Plegisil®, Agrivet, Uppsala, Sweden) in an intramuscular injection and 1 hr later, 10 to 15 mg of mebumalsodium (Mebumal®, ACO, Stockholm, Sweden) intravenously. With additional local anesthesia (Xylocain®, Astra, Södertälje, Sweden) a midline laparotomy was then performed, and the lower ileum was ligated into five or six about 10-cm loops (9–12 cm) with a few centimeters between each. One of the loops was injected in one end with 2 ml of saline, and the remaining loops were injected with 2 ml of toxin in different concentrations. A second ligature was arranged to prevent leakage at the injection site. The abdominal incision was sutured in two layers; the rabbits were left with water ad libitum for 18 to 21 hr and then sacrificed. The abdomen was opened, and the loops were examined for content of fluid.

Titration of neutralizing antibody. Antibodies in serum capable of neutralizing the intradermal PF activity of toxin (3) were titrated as described by Benenson et al. (1).

RESULTS

Neutralizing serum antibody response after vaccination. The capacity of toxin, Formalin-induced toxoid, and heat-induced toxoid to give rise to formation of neutralizing serum antibody was investigated. The response to two subcutaneous injections with optimal doses given 3 weeks apart is shown in Table 1. It is apparent that the toxin was somewhat more immunogenic than the Formalin-toxoid and considerably better than the heat-toxoid in this respect.

The influence of the route of administration of the immunogen was also studied. It was found that, in contrast to the subcutaneous route which regularly led to high titers of neutralizing serum antibody which were further increased after revaccination, immunization intraluminally in the gut only exceptionally induced formation of serum antitoxins in detectable amounts. Thus, in none of the three animals given a single injection and in only one of the three given two injections, such antibodies were registered (Table 2).

In vivo ileal loop protection by vaccination. The minimal toxic dose of the culture filtrate material used for challenge was determined in the ileal loop system. Altogether 15 nonimmunized 7- to 11-week-old rabbits were tested on four different occasions over a period of a month. The results presented in Table 3 show that doses of 4 mg and higher consistently induced accumulation of fluid in the gut and that a dose of 3 mg gave positive loops with about 80% frequency. Based upon these tests, 4 mg and twofold multiples of this dose were regularly used for challenge of the immunized animals. Occasionally 3 and 6 mg were also tested.

As is evident from Table 4, subcutaneous vaccination with toxin induced increased resist-

TABLE 1. *Serum antibody titers after immunization with toxin or toxoid*[a]

Determinations	Titers (after 1st injection)[b]			Titers (after 2nd injection[b])		
	1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks
Toxin	0	279 (27–729)	729 (243–2,187)	4,731 (243–13,122)	6,438 (1,458–13,122)	
Formalin-toxoid	0	0	271 (3–729)	1,701 (729–2,187)	1,701 (729–2,187)	1,701 (729–2,187)
Heat-toxoid	0	0	0	39 (9–81)	135 (81–243)	117 (27–243)

[a] Numbers indicate mean values. Numbers in parentheses indicate range.
[b] Subcutaneous injections.

TABLE 2. *Serum antibody titers after subcutaneous and intraluminal immunization with toxin*[a]

Injections	No. of injections	Titers		
		1[b]	2	3
Subcutaneous	1	0	279 (27–729)	729 (243–2,187)
	2[c]	4,731 (243–13,122)	6,438 (1,458–13,122)	
Intraluminal	1	0	0	0
	2[c]	18 (0–54)	18 (0–54)	

[a] Numbers indicate mean values. Numbers in parentheses indicate range.
[b] Time after injection (weeks).
[c] Three weeks apart.

ance to toxin challenge, whereas the protective effect of such vaccination with formalinized toxoid was less pronounced and with heated toxoid was not ascertained. These experiments, including the challenge tests, were performed concurrently. Two subcutaneous injections of vaccine appeared to be no more protective than one single injection (Table 5), but this comparison refers to nonconcurrently performed experiments.

Also, local intraluminal immunization could induce increased resistance to toxin challenge (Table 5). The resistance after two doses of intraluminal immunization was of the same magnitude as that obtained by the subcutaneous immunization.

Relation between protection and neutralizing serum antibody. The relation between the degree of in vivo protection and the serum titer of the neutralizing antibody at the time for the challenge was in several instances poor, as is apparent in table 5. Thus, a serum titer of 13,122 obtained after two subcutaneous immunizations was associated with no better protection than a titer of 18 obtained by a single subcutaneous injection. Even animals immunized intraluminally in the gut by two injections showed, in spite of absence of demonstrable serum antitoxins, a similar degree of protection as the animals vaccinated twice subcutaneously.

Serum transfer experiments, however, indicated that serum antibodies can contribute to the antitoxic resistance of the gut. Nonvaccinated animals passively immunized with an injection into a marginal ear vein of 8 ml or 2 ml of an antiserum with high titer of antitoxins were protected to some degree, which was not the case with animals transfused with these volumes of preimmunization serum (Table 6).

DISCUSSION

It is evident from the presented results that subcutaneous as well as intraintestinal immunization with toxin can induce increased resistance to challenge with toxin in the experimental model adopted. The values on the size of the increase of the resistance by the immunization deducible in this report (i.e., up to some 5- to 10-fold above that of the control group) might be underestimated, since further work has revealed that the dialysis procedure adopted for the crude toxin before the challenge reduces the toxicity about fourfold but not the capacity to bind antitoxin antibodies. This means that only a minor part of the available antibodies induced by the vaccination might have reacted with molecules with retained toxicity and the rest with detoxified material.

The data also indicate that toxin from the point of protection is more immunogenic than toxoids obtained by Formalin or heat treatment, although it should be emphasized that the study

TABLE 3. *Ileal loop response in unimmunized rabbits to toxin challenge*

Challenge dose (mg)	No. of positive loops (≥0.5 ml/cm)/ tested ones	Volume of positive loops (ml/cm)[a]
1	0/8	
2	3/7	2.1 (1.6–2.8)
3	5/6	1.5 (1.0–2.2)
	11/11	2.4 (2.0–3.4)
8	11/11	2.9 (2.0–5.0)
16	8/8	3.4 (2.0–6.0)
32	6/6	3.5 (2.5–5.5)

[a] Numbers indicate mean values. Numbers in parentheses indicate range.

TABLE 4. *Ileal loop response to toxin challenge in animals immunized with toxin or toxoid*

Immunized[a] with	Animal	Loop response[b] to toxin challenge						Neutralizing serum antibody titer
		3[c]	4	6	8	16	32	
Toxin	1		−		−	++	+++	13,122
	2		−		−	++	+++	4,374
	3		−		(+)	+++	+++	4,374
Formalin-toxoid	1	−	+++	+++	+++	+++		729
	2	−	++	+++	+++	+++		729
	3	−	++	+++	+++	+++		2,187
Heat-toxoid	1	++	−	++	+++	+++		243
	2	−	+++	+++	+++	+++		27
	3	++	+++	+++	+++	+++		81

[a] Two subcutaneous injections 3 weeks apart; challenge 3 weeks after last injection.
[b] Symbols (ml/cm): −, <0.5; (+), 0.5 to 1.0; +, 1.0 to 1.5; ++, 1.5 to 2.0; +++, >2.0.
[c] Challenge dose (mg).

TABLE 5. *Ileal loop response to toxin challenge after subcutaneous and intraluminal immunization with toxin*

Animal	No. of injections[a]	Interval between injections (weeks)	Interval to challenge after last injection (weeks)	Loop response[b] to toxin challenge				Neutralizing serum antibody titer
				4[c]	8	16	32	
1	1		3	−	−	(+)	+++	18
2	1		3	−	−	(+)	+++	162
3	1		3	−	−	+++	+++	486
4	2	3	1.5	−	−	++	+++	13,122
5	2	3	1.5	−	−	++	+++	4,374
6	2	3	1.5	−	(+)	+++	+++	4,374
1	1		3	++	++	+++	+++	0
2	1		3	−	+++	+++	+++	0
3	1		3	+	++	++	++	0
4	1		1	−	++	+++	+++	0
5	1		1	++	+++	+++	+++	0
6	1		1	[d]				0
7	2	2	2	−	−	++	++	54
8	2	2.5	2	−	−	++	+++	0
9	2	2.5	2	−	+	+++	+++	0

[a] The first six were injected subcutaneously; the rest were injected intraluminally.
[b] Symbols (ml/cm): −, <0.5; (+), 0.5 to 1.0; +, 1.0 to 1.5; ++, 1.5 to 2.0; +++, >2.0.
[c] Challenge dose (mg).
[d] Animal died 1 hr after operation.

was performed with very crude toxin. Also with regard to capability to elicit formation of neutralizing antibodies at the serum level, toxin appeared to be more efficient than the Formalin-toxoid and definitely superior to the heat-toxoid. These results are not in agreement with the findings of Feeley and Roberts (8). The discrepancy might possibly be due to the different ages of the immunized animals or to possible differences in storage and handling of the materials used for immunization leading, for example, to different aggregation states of the immunogens. The low age of the animals in the present study might also be responsible for the more pronounced individual variation in antibody response than noted in an earlier study employing adult rabbits (J. Holmgren et al., *to be published*).

Studies of experimental canine cholera have shown a close correlation between the degree of antitoxic immunity and the serum levels of neutralizing antibodies (6). Furthermore, employing isolated ileal segments from immunized

TABLE 6. *Ileal loop response to toxin challenge in animals passively immunized with intravenously administered antiserum*

Passive immunization	Volume (ml)	Animal	Loop response[a] to toxin challenge					
			3[b]	4	6	8	16	32
Non-immune serum (titer[c] <2)	8	1	++	++	+++	+++		
	2	2	++	+++	+++	+++		
Immune serum A (titer 4,000)	8	1	−	−	+++	+++	+++	
	2	3	−	+	++			
	2	4	−	+++	+++	+++	+++	
Immune serum B (titer 6,000)	2	1	−			−		++

[a] Symbols (ml/cm): −, <0.5; +, 1.0 to 1.5; ++, 1.5 to 2.0; +++, >2.0.
[b] Challenge dose (mg).
[c] Neutralizing antibody.

or unimmunized dogs and perfusing them with blood from immunized or unimmunized "pumper" dogs, it was shown that serum antibodies were protective in this system in apparent contrast to locally formed immune factors (5). As pointed out by the authors, however, the test ileal segments were perfused intraluminally with a buffer solution before challenge, and it was not excluded that locally formed antibodies might have been washed out by the perfusate and that this accounted for the absence of locally formed immunity. The data in the present report employing the rabbit ileal loop model are consistent with an antitoxic immunity of dual origin, i.e., immunity mediated by serum antibodies as well as locally formed antibodies. Thus, intravenously injected antitoxins of the IgG class increased the resistance to toxin challenge in nonvaccinated animals, but functional antitoxic immunity was also induced by intratestinal immunization resulting in no or low levels of antitoxins demonstrable in serum. It remains to be settled whether the serum antibodies confer protection while still being in the gut capillaries and tissues or if passage into the gut lumen is essential. Preliminary findings of Burrows et al. (2) showing that protection correlated better with serum titer than with antitoxin titer of ileal loop washings suggest that the local immunity is of less importance than the serum-mediated immunity, but much work remains to establish the relative significance of these two immune entities in protection against asiatic cholera. Recently, enteropathogenic strains of *Escherichia coli* were shown to produce a diarrheogenic exotoxin very similar to that of *V. cholerae*, and such toxins have also been demonstrated from *Shigella dysenteriae* and *Clostridium perfringens* (15). It is therefore likely that intensified research on the problems dealt with in this report might shed light upon immunity against other diarrheal diseases of infectious etiology than cholera.

ACKNOWLEDGMENTS

This study was supported by grant K71-16X-3382-01A from the Swedish Medical Research Council. One of us (J.H.) has research fellowship B72-40P-3591-01 from the Swedish Medical Research Council.

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