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IMMUNOLOGIC INTERRELATIONSHIPS OF COLIFORM HEAT-LABILE AND HEAT-STABLE ENTEROTOXINS

Annual Report

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The goal of these studies is the development of an immunization program to prevent diarrheal disease due to intestinal contamination by enterotoxigenic strains of coliform bacteria. We have demonstrated that (a) active immunization with a purified preparation of the polymyxin release form of *Escherichia coli* heat-labile enterotoxin yields protection against strains of this bacteria which produce this form of toxin; (b) the use of cimetidine renders the peroral route of immunization effective; and (c) peroral, but not parenteral, immunization affords extended protection.

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FORWARD

This annual report describes research investigations conducted during the eleven month period between our previous annual report submitted on February 15, 1978 and January 15, 1979. A considerable portion of the work completed during this period has already been published and in those instances where such is the case, only a brief summary will be presented in this report.

The goals of our research during the second year of support (of which we are half-way through) were: (a) to produce a purified preparation of the polymyxin-release form of *E. coli* heat-labile toxin (LT); (b) to determine optimal routes and schedules for active immunization in rats using this material; and (c) to determine whether such immunization will protect against the water secretory effect of the toxin during in vivo colonization by a toxigenic strain in monocontaminated germfree rats. Projects a and b have been completed and project c is currently in progress.

(1) PROTECTIVE EFFECT OF ACTIVE IMMUNIZATION WITH PURIFIED *Escherichia coli* HEAT-LABILE ENTEROTOXIN IN THE RAT.

Work on this project commenced in the spring of 1978 and the basic aspects of the investigation were completed by September. A report of the results has been accepted for publication in INFECTION AND IMMUNITY and is scheduled to appear in the March, 1979 issue. Copies of the manuscript were submitted with our quarterly report of September 1, 1978.

Basically, this study showed that short-term protection (ie. in rats challenged at one week after completion of boosting) can be achieved by immunization using either the parenteral or oral routes. Immunization exclusively by the parenteral route was highly effective, especially when boosting doses were given with Freund incomplete adjuvant. Parenteral priming followed by intestinal boosting was also effective when the boosts were given directly into the duodenum but not when they were given perorally even after the intragastric administration of bicarbonate. This led us to test the effect of ablating gastric secretions by the use of cimetidine administered intragastrically 2 hours before the peroral boost; this approach resulted in significant protection. We then found that the degree of protection is related to the dosage of the peroral boost: that a 250 µg weekly boost yields more protection than a 50 µg boost. Immunization exclusively by the peroral route (ie. priming and boosting) also yielded significant protection but only when the immunizing dosage was 250 µg per week. (We are currently evaluating whether even larger peroral immunizing dosages in the range of 500 µg will yield a greater degree of protection). In addition to challenge by toxin, which was conducted in all groups, rats immunized exclusively by the parenteral route or boosted perorally using cimetidine were challenged by the instillation of graded concentrations of viable organisms in ligated ileal loops. Both groups of rats were protected against a strain which produces just LT, as well as against a strain which produced LT and the heat-stable toxin (ST), but they were not protected against a strain which produces just ST (see attached Figure 1).

Since completing this initial work, we have continued to explore the optimum approach to immunization in terms of determining (a) which regimen yields the maximum immediate protection and (b) which regimen yields the maximum prolonged protection. In order to accomplish the first goal, we are in the process of reexamining the degree of protection in various immunization groups by extending the challenge concentration out to that dosage which will permit quantitation of the degree of protection

by calculation of the protection index. We are also using this approach to evaluate several different approaches to the various immunization schedules. To cite one example, we questioned as to whether four weekly peroral boosts are necessary after parenteral immunization to yield strong protection. The data obtained, shown in Figure 2, indicate that they are; nevertheless, this does not answer the next question which we will now consider; namely, whether biweekly boosts using larger doses would be equally effective as four weekly boosts.

We have now completed an evaluation of extended protection following immunization by two routes—intraperitoneal priming and boosting (IP/IP) and IP priming and peroral boosting (IP/PO)—using schedules which had achieved strong short-term protection. The results, shown in Figure 3, clearly indicate that peroral immunization is required for prolonged protection. Protection in the IP/IP group was lost within three weeks but it extended during the entire length of the period of study, three months, in the IP/PO group. We will evaluate the length of protection in animals immunized just by the peroral route commencing as soon as we have complete data which identifies the optimum dosage for this approach; this information should be available within the next two weeks.

(2) IMMUNOLOGIC PROTECTION DURING MONOCONTAMINATION BY TOXIGENIC *E. coli* IN GERMFREE RATS.

Our initial observations concerning the suitability of the monocontaminated germfree rat as a model to investigate the pathophysiology of intestinal colonization by toxigenic strains of coliform bacteria were described in last year's annual report. This material is scheduled to be published in the February, 1979 issue of GASTROENTEROLOGY.

By September, we felt that our basic studies concerning optimum approaches to active immunization were sufficiently complete such that we could commence work with the germfree model. To our consternation, we found that the sole vendor of germfree rats (Charles River Breeding Laboratory) was no longer able to provide Sprague-Dawley rats, the strain used in our previous study. They were able to provide Fisher strain rats; however, because these are a different strain with a different plasma osmolality (requiring a modification of the perfusion fluid), we were required to redo all our basic observations in noncontaminated animals and those contaminated by a nontoxigenic strain of *E. coli*.

Preliminary pilot studies indicated that, just as we had found in our previous study using the toxigenic strain of *E. coli* 334, intestinal colonization by strain H-10407 (which is an ST/LT producer and has colonization factor) evokes jejunal water secretion only after several weeks of colonization. For this reason, we focused on ileal transport and have now completely defined the model in terms of contamination by a nontoxigenic and a toxigenic strain (Figure 4). Bacterial colony counts in the ileum have consistently ranged from 10^6 to 10^8 throughout the three week test period in all contaminated animals. Next week we will commence immunization in a group of 10 germfree rats, and the following week in another group of 10, by the IP/PO route using a peroral immunization dosage of 250 μg per week. One week after the final boost (ie. in late February), the immunized rats will be contaminated by strain H-10407 and ileal water transport at two and three weeks after contamination will be evaluated and compared to the values already obtained in unimmunized animals.

(3) ENTEROTOXIGENICITY OF ENTEROPATHOGENIC SEROTYPES OF *Escherichia coli* ISOLATED FROM INFANTS WITH EPIDEMIC DIARRHEA.

This study has been published (Infection Immunity 21:171, 1978) and abstract is appended to this report. Basically, the study showed that strains of enteropathogenic serotypes of *E. coli*, which on epidemiologic groups were clearly the causal agents responsible for outbreaks of infantile diarrhea but which did yield evidence of enterotoxigenicity in conventional assay systems, do produce toxin-like material capable of inducing water secretion when perfused through the rat jejunum. The validity of these observations, and their significance for disease in humans, has been established by publication of work from another laboratory (Lancet 1:1119, 1978) which showed that these strains produce diarrheal disease when administered perorally to human volunteers.

(4) ENTEROTOXIGENICITY OF COLONIZING COLIFORM BACTERIA IN TROPICAL SPRUE AND BLIND-LOOP SYNDROME.

This study has been published (Lancet 2:342, 1978) and abstract is appended to this report. We were able to show that the predominant jejunal coliform flora isolated from 12 patients with tropical sprue consistently elaborated potent toxin material capable of altering intestinal water transport, whereas strains of similar species isolated from the small bowel of persons with the blind loop syndrome did not. This difference in the toxigenicity of these strains may account in part for the different intestinal response in these two disorders to contamination by coliform bacteria.

(5) COMPARISON OF ASSAY OF COLIFORM ENTEROTOXINS BY CONVENTIONAL TECHNIQUES VERSUS IN VIVO INTESTINAL PERFUSION.

These investigations were conducted in collaboration with Dr. Richard Guerrant of the University of Virginia Medical School and with the Center for Disease Control, Atlanta. They were undertaken to compare the results of various assay systems in detecting enterotoxin activity of strains of coliform bacteria. Thirty-six strains of coliform bacteria were tested for enterotoxigenicity by conventional assays, including the Y-1 adrenal and Chinese hamster ovary cell assays for heat-labile (LT) toxin and the suckling mouse assay for heat-stable (ST) toxin, and for the ability of graded concentrations of ultrafiltrate toxin preparation to induce water secretion during in vivo perfusion in the rat jejunum. The ultrafiltrates of all 18 strains isolated from persons with diarrheal disease, including 7 of *Escherichia coli*, 7 of *Klebsiella pneumoniae*, and 4 of *Enterobacter cloacae* contained one (9 strains) or two (9 strains) toxin fractions, resembling either LT or ST in terms of apparent molecular weight and heat-lability characteristics, that induced water secretion at perfusion concentrations of 10 ng per ml or less. Five of the strains of *E. coli* and 2 of *Klebsiella* evoked a positive response in one or more of the conventional assay systems. None of 18 strains isolated from control sources produced similarly potent ultrafiltrate material and all were negative in the conventional assays. These results indicate that many strains of coliform bacteria, particularly of *Klebsiella* and *E. cloacae*, which elaborate potent toxin materials capable of inducing water secretion yield a negative response in conventional assay systems for enterotoxigenicity. A report of this investigation has been submitted to INFECTION AND IMMUNITY.

SUMMARY

Our observations clearly establish the fact that active immunization using a purified form of polymyxin release *E. coli* heat-labile enterotoxin can produce strong immunological protection in rats against challenge by either this toxin itself or viable organisms which produce it. Immunologic protection can be achieved either by parenteral immunization or by peroral immunization if gastric secretion is ablated by the concomitant administration of cimetidine. Immunization by the parenteral approach alone yields only short-lived protection whereas immunization by a combined parenteral-peroral approach arouses prolonged protection. The protective effect of the latter approach during actual chronic intestinal contamination is currently being evaluated in germfree animals monocontaminated by a toxigenic strain of *E. coli*. These observations strongly suggest the feasibility of, and may lead to, a program of prophylactic immunization in humans against diarrheal disease caused by intestinal contamination by LT-producing strains of *E. coli*.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences National Research Council.

PUBLICATIONS

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2. Klipstein FA, Engert RF: Immunological relationship of different preparations of coliform enterotoxins. Infec Immun 21:771, 1978

3. Klipstein FA, Engert RF: Reversal of jejunal water secretion by glucose in rats exposed to coliform enterotoxins. Gastroenterology 75:255, 1978

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6. Klipstein FA: Enterotoxigenicity of *Klebsiella pneumoniae* In McClung HJ (Editor), Etiology, Pathophysiology, and Treatment of Acute Gastroenteritis. Report of 74th Ross Conference on Pediatric Research. Columbus, Ohio, 1978, p. 78-82

7. Klipstein FA, Goetsch CA, Engert RF, Short HB, Schenk EA: Effect of monocontamination of germfree rats by enterotoxigenic coliform bacteria. Gastroenterology 76: In press

8. Klipstein FA, Engert RF: Protective effect of active immunization with purified *Escherichia coli* heat-labile enterotoxin in the rat. Infec Immun In press

9. Klipstein FA: Tropical Sprue. In Principles and Practice of Infectious Disease. Mandell GL, Douglas RG and Bennett JE, Editors. New York, John Wiley and Sons. In press

10. Klipstein FA, Guerrant RL, Wells JG, Short HB, Engert RF: Comparison of assay of coliform enterotoxins by conventional techniques versus in vivo intestinal perfusion. Submitted for publication

Protection Afforded by Immunization with *E. coli* LT Against Various Challenges

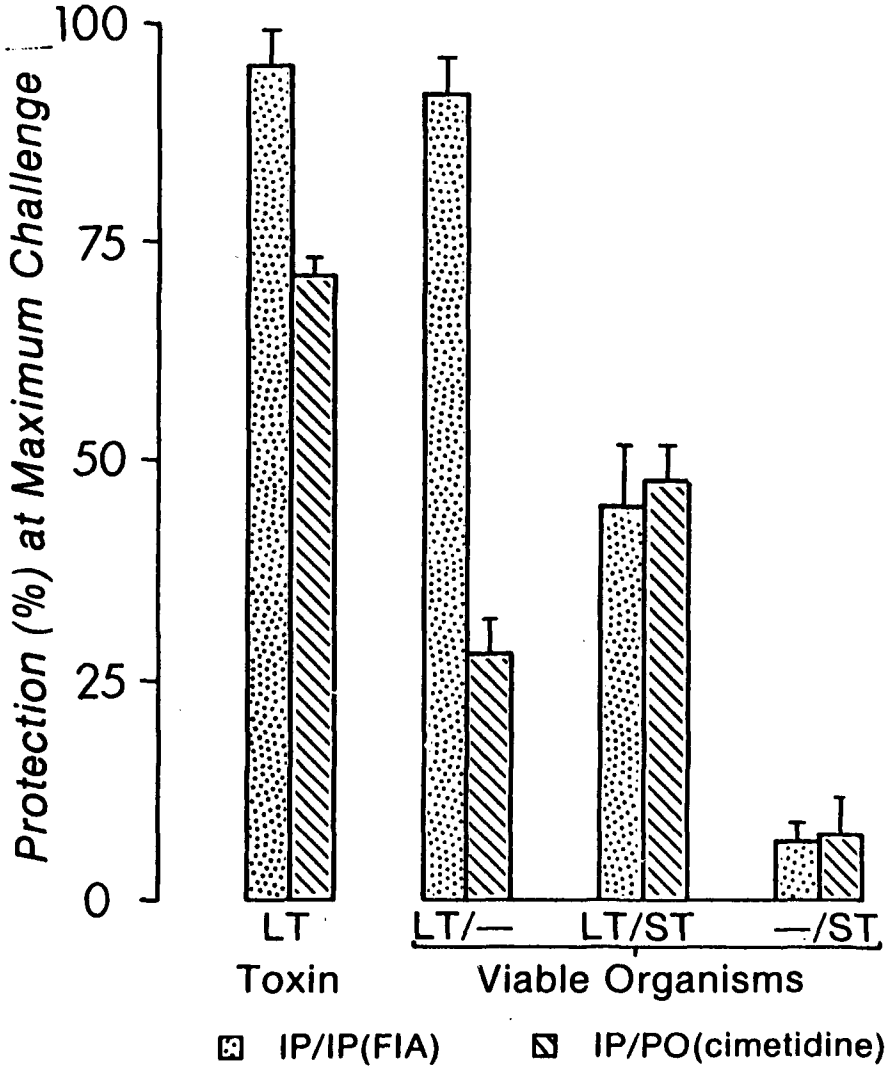

FIGURE 1

Values for the IP/PO route are for boosting at the 50 µg per week dosage.

RELATIONSHIP OF PROTECTION TO THE NUMBER OF WEEKLY PERORAL BOOSTS GIVEN AFTER PARENTERAL PRIMING (IP/PO)

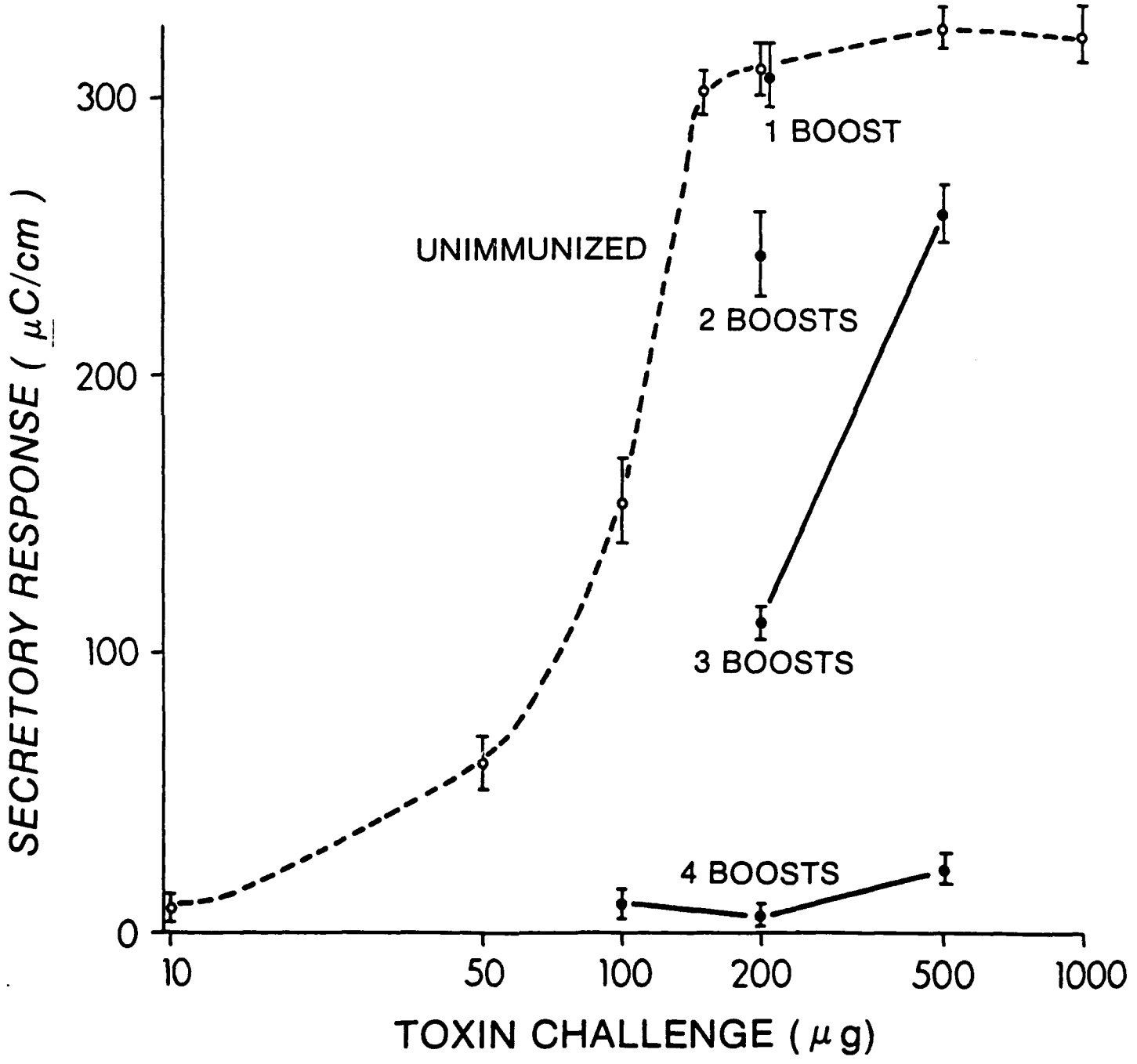

FIGURE 2

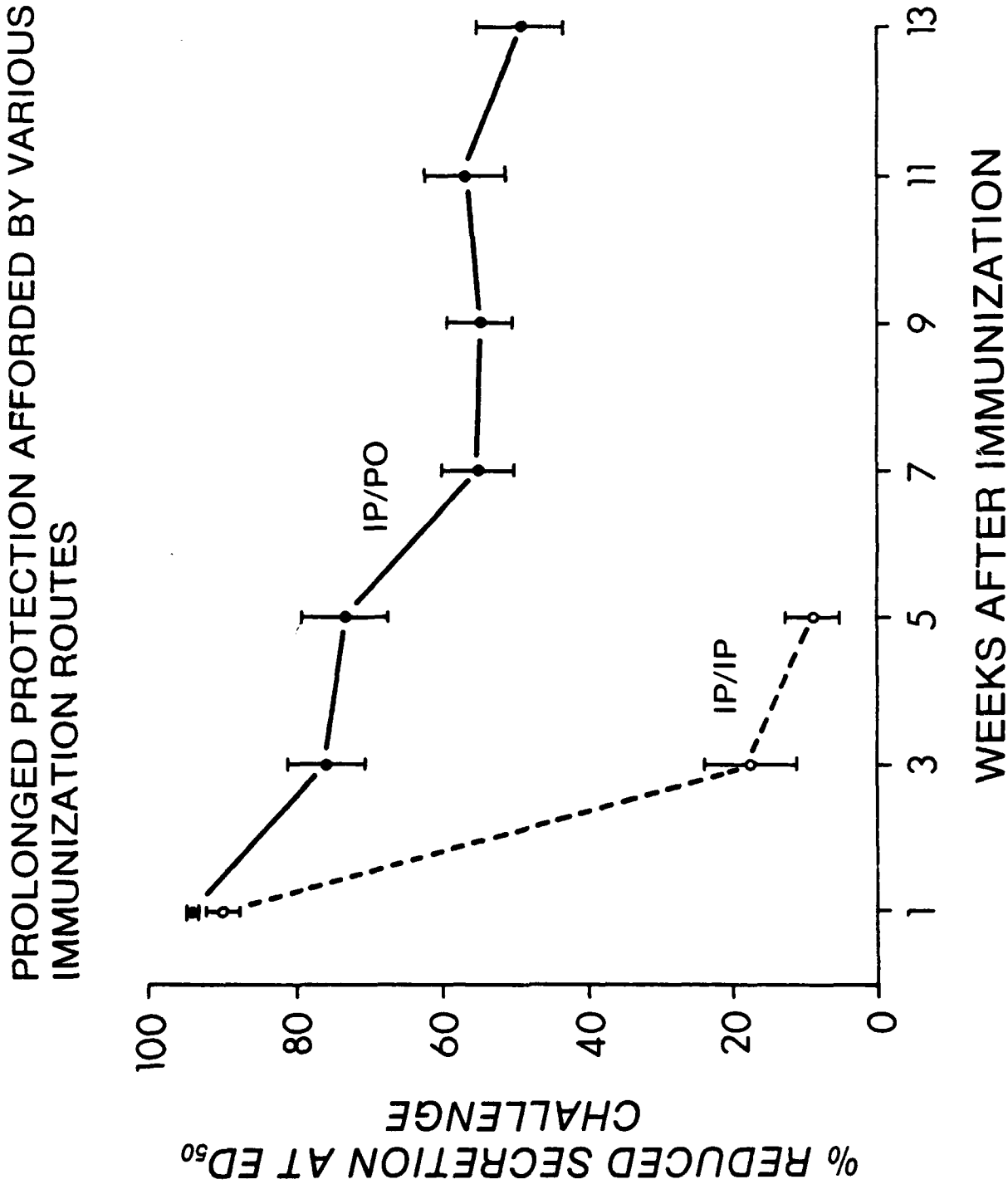
PROLONGED PROTECTION AFFORDED BY VARIOUS IMMUNIZATION ROUTES
% REDUCED SECRETION AT ED_{50} CHALLENGE
100
80
60
40
20
0
1
3
5
7
9
11
13
WEEKS AFTER IMMUNIZATION
IP/PO
IP/IP

FIGURE 3

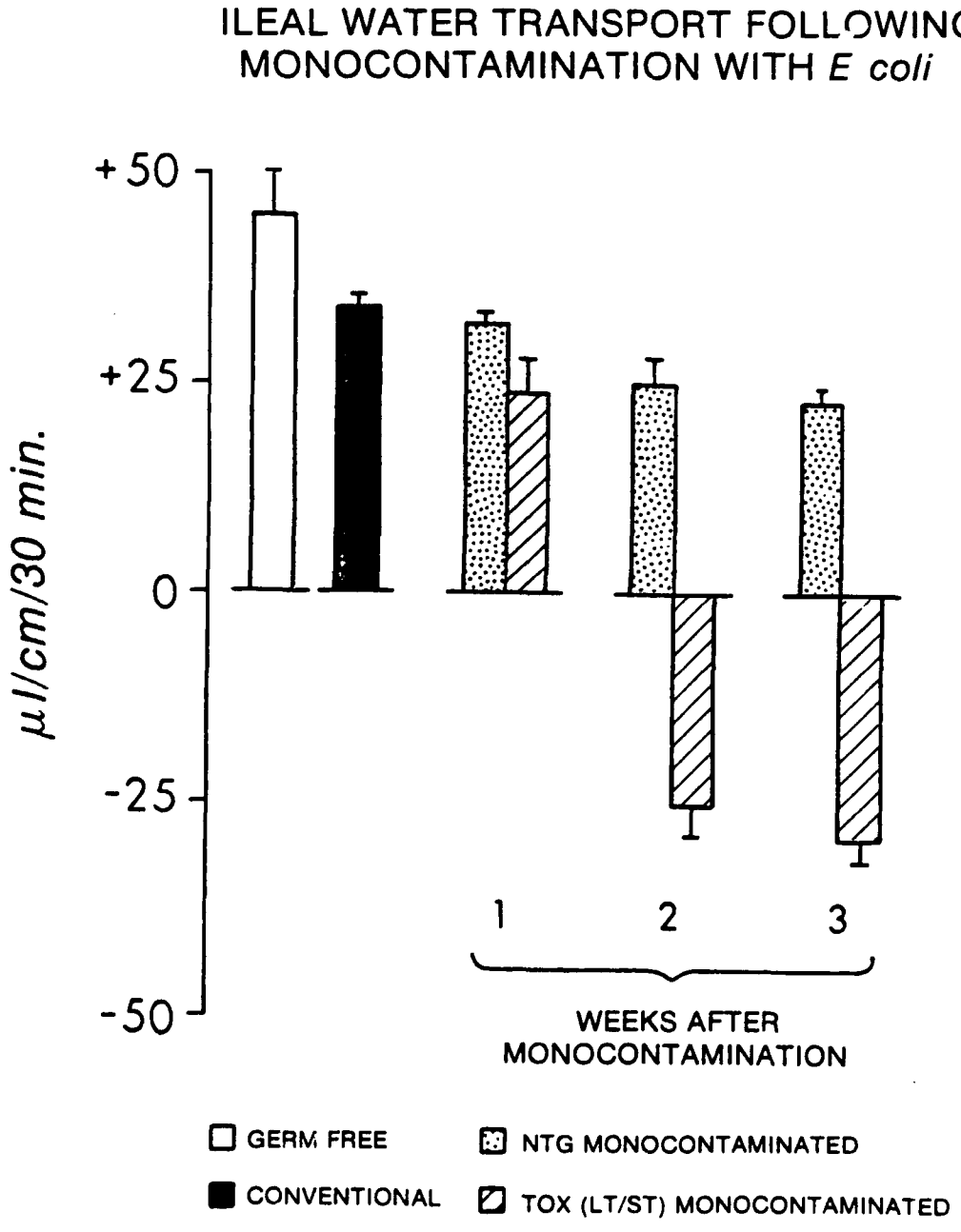
ILEAL WATER TRANSPORT FOLLOWING
MONOCONTAMINATION WITH E coli
+50
+25
0
-25
-50
µl/cm/30 min.
1
2
3
WEEKS AFTER
MONOCONTAMINATION
GERM FREE
CONVENTIONAL
NTG MONOCONTAMINATED
TOX (LT/ST) MONOCONTAMINATED

FIGURE 4

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