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

Annual Report

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**Title:** Immunologic Interrelationships of Coliform Heat-Labile and Heat-Stable Enterotoxins

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**Period Covered:** Jan. 15, 1979 - Jan. 15, 1980

**Summary:**

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 found that immunization with the polymyxin-release form of *Escherichia coli* heat-labile enterotoxin by means of a parenteral prime followed by peroral boosts yields the combination of maximum immediate and extended protection and demonstrated that this immunization regimen is totally protective when photobiotic rats are challenged by intestinal contamination with strains of *E. coli* which produce the heat-labile toxin.
SUMMARY

These investigations have identified the parenteral/peroral route as the optimal route of administration for immunization with *E. coli* LT that provides the combination of maximum immediate and extended protection against challenge with either this toxin or viable strains of LT-producing *E. coli*. Current investigations are addressed at determining whether use of a different form of the LT toxin as the antigen, either the holotoxin or the B subunit or a combination thereof, will provide even more effective protection.
FOREWARD

In conducting the research described in this report the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).
INTRODUCTION

This annual report describes research investigations conducted during the third year of support from the USAMRDC and the Office of Naval Research. For the past two years, our principal efforts have been directed toward ascertaining whether an effective program of immunization against diarrheal disease caused by enterotoxigenic (ETEC) strains of *Escherichia coli* can be developed. The apparent antigenic homogeneity of the heat-labile (LT) enterotoxin produced by heterogeneous serotypes of this organism makes this a logical immunizing agent to evaluate. In last year's annual report we described the results of investigations which indicated that active immunization with this toxin does yield protection in the rat. This study has now been published (Infec Immun 23:592, 1979). We also described our preliminary findings concerning the optimal approach for administration of this immunization program. These studies have been completed during the past year; since the results are in press, they will be presented in summary form.

(1) INFLUENCE OF DOSAGE AND ROUTE OF ADMINISTRATION ON IMMEDIATE AND EXTENDED PROTECTION IN RATS IMMUNIZED WITH *Escherichia coli* HEAT-LABILE ENTEROTOXIN.

Rats were immunized with a purified preparation of the polymyxin-release form of LT, produced as described by Evans et al (J Infec Dis 133:S97, 1976). Parenteral immunizations (IP) were given with Freund complete adjuvant; peroral immunizations (PO) were given by orogastric tube at two hours after the peroral administration of cimetidine in order to ablate gastric secretions. Rats received a prime followed by four weekly boosts and were challenged one week after the final boost (in tests of "immediate" protection) by means of the ligated ileal loop technique. The protection index (PI) was determined by ascertaining protection against challenge with graded dosages of toxin.

(a) Immediate protection against challenge with toxin.

The effect of varying the prime or boosting dosages of the toxin used for immunization on the degree of protection is summarized as follows:

<table>
<thead>
<tr>
<th>Immunization Route</th>
<th>Protection index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100/50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IP/IP</td>
<td>6.4</td>
</tr>
<tr>
<td>IP/PO</td>
<td>3.1</td>
</tr>
<tr>
<td>PO/PO</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Dosage of the prime/boost in micrograms

These observations clearly establish the facts that: (a) increasing the boosting dosage by 5-fold to 250 μg in either the IP/IP or IP/PO routes results in a high degree of protection; (b) increasing the dosage of the prime by 2.5-fold to 250 μg does not yield a further increase in the degree of protection when the larger boosting dose is used in either of these two routes; and (c) a comparable
degree of protection cannot be achieved by the PO/PO route even when the prime and boosting dosages are increased by up to 50-fold. These results exclude the PO/PO route using this antigen as a practical approach for immunization on the basis of the weak protection it affords, and only the IP/IP and IP/PO routes were evaluated in subsequent studies.

(b) Immediate protection against challenge with viable organisms.

Rats immunized by various routes and with different dosages for the boosts were challenged, using the ligated loop technique, with $10^9$ viable organisms of either a strain which produces LT alone or in combination with ST. The results were as follows:

<table>
<thead>
<tr>
<th>Immunization Route</th>
<th>Boost (µg)</th>
<th>Challenge</th>
<th>PB 258 (LT⁺/ST⁻)</th>
<th>H-10407 (LT⁺/ST⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Toxin</td>
<td>V/L</td>
<td>% Reduced</td>
</tr>
<tr>
<td>IP/IP</td>
<td>50</td>
<td>4.6</td>
<td>22 ± 1</td>
<td>92 ± 4</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>9.5</td>
<td>20 ± 9</td>
<td>93 ± 5</td>
</tr>
<tr>
<td>IP/PO</td>
<td>50</td>
<td>3.1</td>
<td>198 ± 1</td>
<td>28 ± 1</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>10.0</td>
<td>66 ± 8</td>
<td>76 ± 3</td>
</tr>
</tbody>
</table>

|                   |           | % Protection index derived from challenge with graded amounts of toxin. |
|                   |           | % Volume/length ratio in microliters per centimeter (mean ± SEM). Values in unimmunized rats were 314 ± 9 for strain H-10407 and 274 ± 18 for strain PB 258. |
|                   |           | Percent reduced secretion (mean ± SEM) as compared to values in unimmunized animals. |

These results indicate that immunization by the parenteral or the parenteral/peroral route provides significant protection against viable organisms of these strains, and that increasing the boosting dosage in the IP/PO route enhances the degree of protection. No protection was evident, at any of the dosages used by either route of administration, against a strain which produces just ST (LT⁻/ST⁺).

(c) Extended protection.

Rats immunized by either the IP/IP or IP/PO routes, using dosages of 100 µg prime and 250 µg boosts, were challenged at bimonthly intervals over a three month period. The results below are for the percentage of reduced secretion (mean ± SEM) in immunized rats compared to the value in unimmunized rats, both challenged with the ED₅₀ dosage of toxin.

<table>
<thead>
<tr>
<th>Weeks after immunization</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>11</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP/PO</td>
<td>94 ± 3</td>
<td>76 ± 7</td>
<td>73 ± 6</td>
<td>55 ± 5</td>
<td>54 ± 4</td>
<td>57 ± 4</td>
<td>49 ± 6</td>
</tr>
<tr>
<td>IP/IP</td>
<td>94 ± 4</td>
<td>25 ± 4</td>
<td>5 ± 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
These results clearly identify the IP/PO route as the only route of administration which yields extended protection. In another group of rats immunized by the IP/PO route, the addition of a fifth boost at two months yielded a value of 93 ± 2 reduced secretion at the ED$_{50}$ challenge at three months. The PI at this time was 6.4. Challenge with viable LT$^+/ST^+$ organisms at three months in this group showed 26 ± 3 percent reduced secretion (as compared to the value in similarly challenged unimmunized rats); this value was significantly less (P < 0.001) than that observed at one week after immunization (78 ± 2 percent reduced secretion).

(2) PROTECTIVE EFFECT OF IP/PO IMMUNIZATION IN GNOTOBOTIC RATS CHALLENGED BY ETEC STRAINS OF Escherichia coli.

The promising results in the preceding studies, which utilized the ligated ileal loop technique for challenge, led us to evaluate immunization by the IP/PO route under conditions which more closely resemble human disease: contamination of the small bowel by ETEC strains of E. coli. Since these strains will not colonize the small bowel of adult conventional animals, we employed the germfree rat as our experimental animal model using techniques which we had developed previously (Gastroenterology 76:341, 1979).

Germfree weanling rats were immunized by the IP/PO route, using a prime of 100 μg and different dosages for the peroral boosts. At the completion of immunization, they were monocontaminated with either an LT$^+/ST^-$ or a LT$^+/ST^+$ strain. Water transport, assessed by the in vivo marker perfusion technique, was determined at weekly intervals thereafter and the results in immunized rats compared to those in unimmunized rats which were contaminated at the same time with either a nontoxigenic (NTG) strain or one of the ETEC strains. The results for monocontamination with the LT$^+/ST^+$ strain are shown below. The results of contamination with the LT$^+/ST^-$ strain were identical with the exception that water secretion was evident only for a one week period after contamination in unimmunized rats.

![Graph showing ileal water transport and bacterial counts](image)
These results clearly demonstrate that this immunization program confers complete immediate protection against intestinal contamination by ETEC strains which produce either LT alone or in combination with ST. They confirm the validity of our results of the ligated loop model and are in accord with previous observations made in that model, namely that the degree of protection is related to the dosage of the peroral boost. The possible factors responsible for the evident protection against a strain which produces ST as well as LT have been discussed in detail in our report on this work, which is in press.

(3) INVESTIGATIONS IN PROGRESS REGARDING IMMUNIZATION WITH *E. coli* LT.

Although the results of the preceding work are encouraging in terms of the development of a program of active immunization against ETEC strains of *E. coli* in humans, certain technical problems remain to be solved. (i) We have found that five immunizations (prime and boosts) are required to achieve a significant degree of extended protection; this number is impractically large. (ii) The degree of protection against viable organisms in rats challenged at three months after immunization was suboptimal. (iii) This immunization program does not yield protection against strains which produce just ST. We are currently working to develop approaches which could solve the first two problems.

(a) Effect of the dosage of the prime.

In our previous studies, we showed that increasing the boosting dosage yielded a higher degree of protection; we gave short shrift to the effect of the priming dosage, however. The latter may be of importance since such has been shown to be the case during immunization with various forms of cholera toxin/toxoid (J Exp Med 195:266, 1978). We are therefore in the process of evaluating this with the polymyxin-release form of LT.

Studies regarding this for the IP/IP and IP/PO routes are in progress. Those for the PO/PO route are completed. They indicate that increasing the prime above 100 µg has no effect. Increasing the priming dosage by ten-fold to 1000 µg and administering this either perorally with cimetidine or directly into the duodenum yielded no increase in the PI. Nor did adding crude toxin (in the form of whole cell lysate) to 100 µg of LT increase the degree of protection. Thus our data shows that exclusively peroral immunization with this antigen fails to provide strong protection under any circumstance.

(b) Effect of different toxin forms as the antigen.

The 30,000 dalton polymyxin-release form of LT which we have used for immunization probably consists of one A and one B subunit (J Infec Dis 133: S97, 1976). Recent studies have shown that the LT holotoxin consists of one A and about six B subunits (Infec Immun 24:760, 1979). Investigations with various forms of the cholera toxin have shown that the B subunit is a highly potent immunogen (Nature 269:602, 1977). It would seem probable, therefore, that we have been using an antigenically suboptimal form of LT for immunization and that use of either the LT holotoxin or B subunit will provide more effective, extended protection.

To evaluate this, we have produced holotoxin by the method described by Clements and Finkelestein (Infec Immun 24:760, 1979) and are currently comparing
the protective effect of immunization with this material to that achieved using the polymyxin-release form of LT. We plan to commence making LT B subunit early next year. Preliminary immunization experiments using holotoxin made in our laboratory and B unit given to us by another laboratory suggest that both are more effective immunogens when given by the IP/PO route. The following preliminary results are for immunization using a 100 μg prime and 50 μg boosts.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Route</th>
<th>PI</th>
<th>Route</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymyxin-release</td>
<td>IP/IP</td>
<td>6.4</td>
<td>IP/PO</td>
<td>3.1</td>
</tr>
<tr>
<td>Holotoxin</td>
<td>IP/IP</td>
<td>6.4</td>
<td>IP/PO</td>
<td>6.2</td>
</tr>
<tr>
<td>B subunit</td>
<td>IP/IP</td>
<td>5.2</td>
<td>IP/PO</td>
<td>7.6</td>
</tr>
</tbody>
</table>

(4) IMMUNOGENICITY OF OTHER COLIFORM ENTEROTOXINS.

During the first year of this contract, we showed that crude heat-labile enterotoxin preparations (whose toxin activity could be detected only by their ability to induce water secretion as tested by in vivo marker perfusion in the rat jejunum) present in whole cell lysates (WCL) of strains of *Klebsiella pneumoniae* or *Enterobacter cloacae* share an immunologic relationship with a heat-labile toxin present in the same form of WCL preparation from an ETEC strain of *E. coli* as well as with cholera toxin. These observations were made by ascertaining passive protection of specific hyperimmune serum on the ability of the various toxins to induce fluid secretion as ascertained by in vivo perfusion (Infec Immun 18:110, 1977; 21:771, 1978).

The following year, we showed that strains of enteropathogenic (EPEC) *E. coli* which do not activate routine assay systems (Y1 adrenal, CHO, suckling mouse) for LT and ST also elaborate enterotoxin material present in WCL which also can only be detected by assay of its effect on water transport by in vivo perfusion (Infec Immun 21:171, 1978). It seemed important to determine whether the EPEC enterotoxin material present in WCL is antigenic and whether it bears any immunologic relationship to conventional *E. coli* LT (which stimulates routine assay systems) from an ETEC strain. In the present study, we evaluated immunologic relationships by means of determining the effect of active immunization. Weanling rats were immunized, by the IP/PO route, with WCL preparations from an EPEC and an ETEC strain as well as with the purified polymyxin-release LT from the ETEC strain. They were then challenged either with 10⁹ viable organisms of these strains or with the ETEC LT toxin using the ligated ileal loop technique.

The results are shown in the Table on the following page. Immunization with the WCL toxin preparations of either the EPEC or ETEC strain conferred protection against challenge with viable organisms of both strains; immunization with a similar preparation from a nontoxigenic strain did not yield protection. Immunization with either the WCL or purified LT toxin from the ETEC strain afforded protection against challenge with the ETEC LT toxin, but immunization with the EPEC WCL preparation did not. The antigenicity of all of the toxin preparations was destroyed by heat-treatment. Possible contributory protective effects of somatic or colonization factor (CFA) antigens present in the WCL were excluded by the findings that protection was afforded against a heterologous somatic serotype, ileal bacterial counts were not reduced in protected animals, and WCL preparations of strains containing or lacking CFA yielded equal protection.
These observations indicate that the heat-labile enterotoxin of EPEC strains is antigenic and is immunologically related to a heat-labile toxin present in similarly prepared material from an ETEC strain but not to the conventional LT toxin of ETEC strains. They suggest that the WCL preparation of the ETEC strain contains two heat-labile enterotoxins, one of which is conventional LT and the other of which resembles the EPEC toxin.

<table>
<thead>
<tr>
<th>Immunization*</th>
<th>Viable EPEC</th>
<th>Viable ETEC</th>
<th>ETEC LT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>Protection</td>
<td>Protection</td>
<td>Protection</td>
</tr>
<tr>
<td></td>
<td>Protection</td>
<td>Bacteria</td>
<td>Bacteria</td>
</tr>
<tr>
<td>NTG</td>
<td>WCL</td>
<td>1 ± 1</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td>EPEC</td>
<td>WCL</td>
<td>89 ± 2</td>
<td>5.8 ± 0.1</td>
</tr>
<tr>
<td>EPEC</td>
<td>WCL(H)</td>
<td>4 ± 2</td>
<td>8.5 ± 0.1</td>
</tr>
<tr>
<td>ETEC</td>
<td>WCL</td>
<td>87 ± 3</td>
<td>6.9 ± 1.3</td>
</tr>
<tr>
<td>ETEC</td>
<td>WCL(H)</td>
<td>1 ± 1</td>
<td>4.9 ± 0.7</td>
</tr>
<tr>
<td>ETEC</td>
<td>LT</td>
<td>0</td>
<td>7.0 ± 1.3</td>
</tr>
<tr>
<td>ETEC</td>
<td>LT(H)</td>
<td>3 ± 2</td>
<td>4.2 ± 0.3</td>
</tr>
</tbody>
</table>

* (H) signifies that the preparation was heated prior to use as an antigen.
† Mean ± SEM percent reduced secretion in ligated loops of immunized rats as compared to the value in unimmunized animals similarly challenged with either a 15-fold concentration of bacteria or 200 μg of ETEC LT.
‡ Mean ± SEM Log₁₀ organisms per ml recovered from the ileal fluid. Bacterial counts recovered from unimmunized rats were 6.9 ± 2 with the ETEC strain and 7.2 ± 0.5 with the EPEC strain.
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