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The aim of this research was to build immunogens susceptible of inducing a response against enterotoxigenic E.coli (ETEC) following oral administration. The antigenic determinants were peptides copying structures of the colonization factor antigens (CFA), the carriers were either the β-chain of cholera toxin or a synthetic copy of one of its fragment. This fragment is known to bind in the Payer's patches the GM1 ganglioside which represents the toxin receptor. Two different E.coli peptides have been analyzed by HPLC. Eight different conjugates have been prepared using glutaraldehyde or carbodiimide. The two peptides have been linked to MDP-Lys, 6-O-succinyl murabutide, β-chain of cholera toxin and the GM1 ganglioside binding peptide. After analysis, these conjugates have been sent to Dr. Reid to be tested in in vitro systems. They have been administered to mice orally. The antibody response has been studied in sera. Only a slight anti-β-cholera toxin response has been obtained. It would be advisable to increase the dosage of immunogens.

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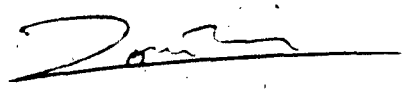

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TABLE OF CONTENTS

REPORT

Introduction.

Oral immunization employing live organisms is known to induce protection against mucosal infections (1) and to evoke IgA responses. The same route of immunization is less successful when killed organisms or purified fractions are used. This situation is true for enterotoxigenic E.coli strains (ETEC) which are responsible for severe gastrointestinal disorders principally in infants and young animals but also in adults. These disorders, which are caused either by a thermolabile enterotoxin (LT) or by a small heat stable toxin (ST) are known to considerably impede military mobilization and deployment (2-5). The major virulence factors of ETEC have been shown to be colonization factor antigens (CFA : CFAI, CFAII and PCF 8775) (6-12). These CFA which are responsible for the attachment of bacteria to the mucosa of the small intestine and for its colonization are shared by several serogroups of E.coli. These organisms have been shown to be good candidates for potential use in vaccines (6-19). It would be advantageous to be able to induce protective mucosal immunity to these antigens. This could be achieved by combining several techniques presently available. These include the use of carriers which are able to specifically stimulate immune responses in Peyer patches and the use of adjuvants capable of modulating the isotypic pattern of antibody synthesis.

Muramyl dipeptide (MDP, Nac-Mur-L-Ala-D-isoGln) represents the minimal structure capable of substituting for Mycobacteria in Freund complete adjuvant (FCA (20-21). The original molecule and several of its analogs or derivatives are adjuvant-active in saline (22-23) and one of them has been shown adjuvant-active in humans when associated with tetanus toxoid (24). These molecules are also capable of modulating the class of antibodies synthesized during a humoral response (25-26). For example, increased IgA antibody titers in both

serum and saliva have been induced in rats given Streptococcus mutans antigens and MDP by gastric intubation. These rats were shown to be protected against caries induced by a virulent challenge of virulent S.mutans (27). Similar results were also observed by Taubman et al. (28) and the oral administration of bovine serum albumine into liposomes containing a lipophilic derivative of MDP has been also reported (1). It has been postulated that potentiation of IgA response after oral administration of antigen with MDP could be mediated by cells present in Peyer's patches since MDP has been shown to promote the in vitro response of these cells to sheep red blood cells (29).

It is well known that oral immunization can lead to the production of systemic tolerance in response to the parenteral injection of specific antigen (30). However, it is possible to trigger mucosal immunity by using appropriate carriers, for example, the utilization as carrier of either whole cholera toxin (CT) or its β-chain subunit (31-32) or the heat-labile enterotoxin of E.coli LT (33) can breck oral tolerance and lead to the synthesis of antibodies mainly secretory IgA. This property of CT and LT has been related to their ability to bind to the GM1 ganglioside which is the receptor for both toxins (34-35). It is of interest to note that both enterotoxins share antigenic determinants (37). The region of the β-chain of CT responsible for the binding has been synthesized and shown to bind the GM1 ganglioside. This peptide will be referred to as GM1 BP (30-50). Its elongation by an unrelated octopeptide does not affect its binding capacity (38). Moreover, this construct has been shown to be immunogenic and antibodies directed against the octopeptide have been obtained, in an in vitro system. The use of MDP-Lys either free and mixed with the conjugate or, moreover, covalently linked to it has resulted in a greatly increased response (unpublished results).

Synthetic peptides attached to appropriate carriers, polymerized or free in association with effective adjuvants have been shown to induce protective responses against parasitic (39-40), viral (see in 41-42) and bacterial antigens (43-45). Synthetic cholera toxoid (46) and also anti-ETEC Coli vaccines (47) have been synthesized and a preliminary trial has been successfully conducted in humans employing a

construct containing a synthetic heat-stable enterotoxin linked to B subunit of LT (48). This approach will also be considered for preparing anti-CFA vaccines which could be used alone or associated with preparations containing other E.coli antigens as suggested by Ahren and Svennerholm (49). The amino acid sequence of CFA1 is now available (50) as well as the sequence of the CS4 subunit of PCF 8775 (Andrews T., Reid R.J., Seid R.C., Wolf B. and Boedeker E.C., unpublished results) or the sequence of AF/RI antigen (Andrews T., Reid R.H., Seid R.C. and Boedeker E.C., unpublished results). AF/RI antigen is the species specific colonization factor the RDEC-1, rabbit E.coli (51). Experimental models for protection against RDEC-1 have now been well established (51-52) and predictions have been made on the potential immunogenic determinants (54).

Taken together the data indicates that a) the combined use of carriers (either natural or synthetic) binding to the GM1 ganglioside site and use of appropriate MDP derivatives could allow the preparation of orally active vaccines; b) that CFA could provide protective antigenic structures to be included in anti-ETEC synthetic or semi-synthetic vaccines.

Materials.

Immunogenic molecules

We have received from Dr. Reid (Chief Immunology Section, Department of Gastroenterology, Walter Reed Army Institute of Research) the three following synthetic peptides :

1. N-terminal peptide (residues 1-13) of the CFAI fimbrial protein from human enterotoxigenic E.coli strain, Val-Glu-Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro.

2. N-terminal peptide (residues 1-8) of the AF/RI pilus protein from rabbit RDEC-1 E.coli strain, Ala-Gly-Gly-Asp-Val-Glu-Phe-Phe.

3. Peptide 30-50 of the cholera toxin β-chain, Ser-Leu-Ala-Gly-Lys-Arg-Glu-Met-Ala-Ile-Ile-Thr-Phe-Lys-Asn-Gly-Ala-Thr-Phe-Glu-Val which has been found to bind the GM1 ganglioside.

The cholera toxin β-chain has been purchased from Sigma Chemical Co.

Adjuvant molecules

MDP analogues which can be linked to the immunogen have been chosen. In our proposal we had planed to use MDP-Lys and ε-amino caproic murabutide. The first compound has been previously described to exhibit an enhanced adjuvant activity following its coupling to an immunogen sucn as a protein or a synthetic peptide. The ε-amino caproic murabutide can also be conjugated and derived from a non-pyrogenic molecule murabutide (55) which has undergone clinical trials and has been found active when associated with a tetanus toxoid vaccine (24). As will be explained later another molecule had to be used to obtain a sufficient yield of coupling and the conjugate which was used in the experiment murabutide was linked to the peptide through a 6-O-succinyl murabutide derivative.

Experimental Methods and Results

HPLC analysis.

The three peptides have been analyzed by high performance size exclusion chromatography using a LKB high performance liquid chromatography equipment.

The diagrams of elution indicate that the peptides present a very high degree of purity.

Dosage of MDP.

The method of Reissig (56) has been used. It allows to evaluate specifically the N-acetyl group substituted in the 2-position of the muramic acid. The reagents are potassium tetraborate at 2% and the Ehrlich reagent (containing dimethylaminobenzaldehyde in acetic acid and HCl). One hundred microliters of the solution containing 10 to 300 µg of MDP/ml are added to 100 ml of tetraborate in a boiling bath for 5 min. After cooling the mixture, 900 µl of Ehrlich reagent diluted to the 1/8 by acetic acid are added for 30 min at 37°C. The absorbance is read at 585 nm. The MDP content of the experimental samples is evaluated using a standard curve which is included for each dosage.

Conjugation procedures.

Depending upon the chemical groups available for coupling, either carbodiimide or glutaraldehyde have been used.

- Conjugation of N-ter (1-8) to cholera toxin β-chain.

The peptide (3.5 mg) has been dissolved with 1 mg of cholera toxin β-chain in 2 ml of 0/M sodium bicarbonate pH 8. After 1 hr, glutaraldehyde (25% in water, grade J, Sigma Chemical Co.) has been added to a final concentration of 2.63mM with continuous stirring at room temperature. After 5 days the resulting mixture has been dialyzed exhaustively against a phosphate buffered saline.

- Conjugation of N-ter (1-8) to the GM1 binding peptide.

The peptides 3.5 mg and 4.2 mg respectively have been conjugated by glutaraldehyde under the conditions described above.

- Conjugation of N-ter (1-8) to murabutide.

In a first set of coupling $\mathcal{E}$-amino caproic murabutide has been conjugated using glutaraldehyde. The analysis of the conjugate for its N-acetyl-muramic acid content showed that the yield of coupling had been very low since less than 0.1 mg of murabutide per mg of

conjugate could be evidenced. A second set of experiments was carried out using a 6-O-succinyl murabutide. Since the chemical group available is a carboxyl, the coupling was performed through carbodiimide and an excess of MDP was used, 10 mg for 3.5 mg of peptide. The materials were mixed in 2 ml of 0.1M 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide HCl at pH 5 for 10 hr at room temperature. Then, an equal volume of 1.0M glycine was added to stop the reaction, and left overnight at 4°C. The resulting conjugate was dialyzed against phosphate buffered saline. It was found to contain 1 mg of murabutide for 1.5 mg of peptide.

- Conjugation of N-ter (1-8) to MDP-Lys.

  This coupling was performed using glutaraldehyde. The concentrations of material and the conjugation procedure were as above. The conjugate contained 100 µg of MDP for 0.5 mg of peptide.

- Conjugation of N-ter (1-13) to cholera toxin β-chain and to the GM1 binding peptide.

  These couplings were performed as for the N-ter (1-8) peptide.

- Conjugation of N-ter (1-13) to 6-O-succinyl murabutide and MDP-Lys.

  Coupling was carried out as described. The conjugates were as follows : N-ter (1-13) to murabutide contained 150 µg of murabutide for 500 µg of peptide and the N-ter (1-13) MDP-Lys contained 150 µg of MDP for 200 µg of peptide.

Immunization.

The 8 conjugates have been sent to Dr. Reid, Immunology Section, Dept. of Gastroenterology, to be be utilized in in vitro systems of immunization. They have been also used to immunize mice orally. Mice were C3H/He animals purchased from Harlan Laboratories (Madison, WI). They received at 1 month interval two oral immunizations.They were bled by the orbital plexus 14 days after the first immunization, and 7 and 14 days after the secondary immunization. Groups of 10 animals were as follows : 1) controls, 2) N-ter 1-8 cholera toxin β-chain, 3) N-ter 1-8

cholera toxin + 100 µg MDP, 4) N-ter 1-8 GM1 binding peptide 100 µg, 5) N-ter 1-8 GM1 binding peptide 100 µg MDP, 6) N-ter 1-8 murabutide 100 µg, 7) N-ter 1-8 MDP-Lys 100 µg. The same groups were used to study the immunogenicity of the N-ter (1-13) containing conjugate.

ELISA Method.

Sera were tested by ELISA. Titer plate wells (Nunc immunoplates) were coated with 10 µg of peptide or 5 µg of cholera toxin β-chain per ml. After 2 hr at 37°C, they were washed and incubated with serial dilutions of sera at the same temperature. They were washed again and incubated for another hour with a rabbit anti-mouse immunoglobulin peroxidase conjugate (Miles Laboratories, Naperville, IL). After washing the substrate solution containing 50 mg O-phenylene-diamine (Sigma) in 100 ml of 0.05M citrate/phosphate buffer, pH 5.2, and 20 µl of H_2O_2 (35%) was added. The enzyme reaction was stopped after 10 min by 12.5% (vol/vol) sulfuric acid. Absorbances were read at 492 nm on a Titertek Multiskan ELISA reader (Flow Laboratories, Rockville, MD).

The results obtained showed that a low anti-cholera toxin β-chain could be observed in the two groups treated respectively with the N-ter (1-8) cholera toxin β-chain and N-ter (1-13) cholera toxin β-chain associated with adjuvant as antibodies were found to rank between < 100 to 400. No anti-peptide antibodies could be detected.

This negative result concerning the response to the peptides can be explained as follows :

a) The antibody response might have been evoked only in the secretory system. Further experiments should have been performed to assess or invalidate this tempting hypothesis.

b) The peptides on the plates present a different conformation from their conformation in the conjugate. To control this point, it would have been interesting to prepare anti-peptide sera by coupling each peptide to a potent carrier such as tetanus toxoid and to hyper-

immunize mice by the parenteral route so as to have the best chances of raising high titers of anti-peptide antibodies. These antibodies would have been used to test various conditions of plate coating. Eventually the peptides, since they are short, might have to be conjugated to another carrier such as bovine serum albumine or multi-poly-L-ala-poly-L-Lys chain (A--L) to allow the binding to their specific antibodies. These assays could not be carried out due to the small amounts of peptides available.

c) It cannot be ruled out that the immunogenic determinants of these two peptides have been masked by the coupling. To rule out this possibility it would be appropriate to test their immunogenicity after parenteral administration and to modify the conditions of coupling according to the results obtained.

d) It is also possible that the immunization by the oral route requires higher dosages of conjugate than those used in these experiments. Once again, sufficient amounts of material were not available to repeat the assay using higher dosages.

Concluding remarks.

These experiments have shown that conjugates can be built associating the E.coli peptides to appropriate carriers. Conjugates with built-in adjuvanticity have been also obtained. The low response against the cholera toxin β-chain obtained in the two groups treated with MDP is encouraging. As said previously, nothing can be concluded at the present stage from the negative results observed against the peptides.

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