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Peptides as Immunogens

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Preface

The humoral response of the immune system to a foreign antigen usually requires the recognition of two antigenic determinants. The one, called the carrier, is recognized by T-lymphocytes, the other, called the hapten, by B-lymphocytes. As a consequence, T- and B-lymphocytes proliferate, B-lymphocytes produce hapten-specific antibodies, and the system develops memory to the antigens. It was long thought that antigens would form a bridge to mediate the cooperation of T- and B-lymphocytes. However, it now appears that antigens are broken down to fragments which then act as carrier determinants for T-lymphocytes.

The cells which originally process antigen are called antigen-presenting cells. They have phagocytic properties. They can take up and degrade antigens, in the case of proteins to peptides.

The peptides of protein antigens reappear on the surface of the antigen-presenting cells, where they must become associated with membrane proteins encoded by genes of the major histocompatibility complex (MHC) in order to be recognized by T-lymphocytes. To activate helper T-lymphocytes which cooperate in antibody responses, MHC class II molecules have to be expressed on the surface of the antigen-presenting cells. Once T-lymphocytes have become activated, they are ready to cooperate with B cells.

Antigen-presenting cells are macrophages but can also be B-lymphocytes. In the latter case, a protein antigen can be taken up through binding of one of its heptenic determinants to the hapten-specific antibody displayed on the surface of the B cells. It is then processed and a peptide presented on the surface of the B cells as a carrier determinant in conjunction with class II MHC encoded membrane proteins. T-lymphocytes specific for this peptide-MHC class II complex can, then, recognize the B cell, cooperate with it, and induce it for hapten-specific antibody secretion. The remarkable consequence of this view of T-cell-dependent activation of antibody production by B cells is the realization that processed antigens, e.g., peptides, are separate

physical entities of antigens that have become specifically separate from haptens. Peptides should, therefore, be able to act as carrier determinants which can be used to selectively prime T-lymphocyte compartments of the immune system without stimulating B-lymphocytes.

This selective priming of parts of the immune system is likely to offer new practical approaches for immunizations and, thus, for the development of vaccines.

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Peptides as Immunogens: Prospects for Synthetic Vaccines

R. ARNON

1 Introduction

The basis for the utilization of synthetic peptides as immunogens was laid during our early studies on the antigenicity of proteins. The initial findings that covalent attachment of tyrosine oligopeptides to gelatin by polymeric techniques resulted in augmentation of its immunogenicity (SELA and ARNON 1960) led us to the synthesis of both linear and branched polymers of amino acids, capable of initiating an immune response (SELA et al. 1962). The availability of these synthetic antigens permitted a systematic elucidation of the molecular basis of antigenicity, including the role of such variables as chemical composition, size, shape, accessibility of epitopes, electrical charge, optical configuration, and mainly spatial conformation in rendering a molecule immunogenic and in dictating its antigenic specificity (SELA 1969).

In 1971 we first showed that it is possible to synthesize a peptide which elicits antibodies capable of recognizing a native protein (ARNON et al. 1971). The epitope in this case was the "loop" of hen egg-white lysozyme, demonstrated previously to be a conformation-dependent immunodominant region of the protein (ARNON and SELA 1969). As a corollary, we suggested that by adequate molecular engineering it should be possible to design synthetic materials that would elicit antiviral immunity, leading eventually to multivalent synthetic vaccines (ARNON 1972).

The rapid development of DNA cloning and sequencing techniques which facilitate protein sequencing has made the synthetic approach more feasible and practical than before. Primary sequences are available today for many proteins, enabling the synthesis of any selected region, using either chemical

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laboratory techniques, which are advisable in the case of short peptides, or genetic engineering procedures in the case of longer protein segments, subunits, or even whole proteins. Moreover, the synthetic approach should allow the choice of carrier as well as adjuvant, the goal being the production of multivalent vaccines with built-in adjuvanticity.

2 Identification of Antigenic Determinants

Macromolecular antigens usually express a large number of possible antigenic determinants, or epitopes, that dictate their antigenic specificity. However, only a limited number of the potential antigenic sites are important for immunogenicity, namely, immunodominant ones. In the case of an antigen that possesses biological activity, even fewer epitopes are involved in the neutralizing immune capacity.

It is useful to distinguish between two types of epitopes – *sequential* and *conformational* (SELA 1969). Whereas a sequential determinant is viewed as a sequence of several residues in its unfolded form, a conformational determinant is defined by a number of residues that are maintained in a particular conformation, to which long-range interactions of the levels of secondary, tertiary, and quaternary structure contribute as well. A parallel distinction introduced by ATASSI and SMITH (1978) differentiates between *continuous* and *discontinuous* determinants. A continuous determinant is defined as a sequence of residues exposed at the surface of a native protein, whereas a discontinuous one consists of the juxtaposition in space of residues that are not adjacent in the primary structure.

When one is dealing with protein antigens, it seems that the parameters which are the most influential in contributing to the immunological properties are spatial conformation and accessibility. Accessibility is crucial since the interaction of any epitope with either the antibodies or the specific receptors on the immunocompetent cells necessitates its exposure on the antigenic molecule. As a result, antigenic determinants were identified in many native proteins as segments that are positioned at "corners" of the folded polypeptide chain and fully or partially exposed on the surface of the protein molecule (ATASSI 1975). Hence, the probability that a particular region contains surface residues provides one way of predicting the antigenic structure.

Spatial conformation has long been known to play a decisive role in determining antigenic specificity. There is cumulative evidence that a drastic change in antigenic properties occurs upon denaturation of native proteins or unfolding of their polypeptide chains (BENJAMINI et al. 1972; ARNON 1974; CRUMPTON 1974), or even as a result of more subtle conformational alterations (CRUMPTON and WILKINSON 1966). This has led to the conclusion that most of the antigenic determinants of proteins are conformational determinants. Antibodies specific toward such determinants will not necessarily react with isolated peptides derived from the molecule. It is, therefore, of interest that many investigators have been successful in raising antipeptide polyclonal antisera that recognize

native protein (e.g., ARNON et al. 1976; LERNER et al. 1981; GREEN et al. 1981). This high rate of success may partly be attributed to the fact that the relative flexibility of peptides in solution can be mimicked by local disorder in short segments of the protein. Indeed, it was shown recently that the majority of continuous epitopes of tobacco mosaic virus (TMV) protein, myoglobin, lysozymes, and myohemerythrin correspond to surface regions in those proteins that possess a high segmental mobility (WESTHOF et al. 1984; TRAINER et al. 1984). It is possible that segmental motion in proteins provides the necessary degree of conformational similarity with isolated peptides and that this ensures sufficient antigenic cross-reactivity between the intact molecule and an excised fragment. This is true, however, mainly for relatively short peptide segments. When longer regions are concerned, they usually consist of a more rigid structure and the right conformation is essential for the specificity (WESTHOF et al. 1984; SHAPIRA et al. 1985).

Identification of antigenic determinants can be made by fragmentation of the native protein, either by chemical cleavage or controlled proteolysis. The resultant fragments are then screened for immunologically active components that can bind to antibodies and interfere with their interaction with the intact antigen. Data obtained by those methods have resulted in the identification of antigenic deter nants in several protein antigens such as TMV protein (BENJAMINI et al. 1964; ALTSCHUH et al. 1983), sperm whale myoglobin (ATASSI 1975) or hen eggwhite lysozyme (SHINKA et al. 1962; ARNON and SELA 1969). Alternatively, monoclonal antibodies with neutralizing activity could be useful in identifying relevant antigenic sites. This approach is particularly useful for identification of determinants involved in some biological phenomena, e.g., virus neutralization. An illustrated example is the localization of the various antigenic sites on influenza hemagglutinin (HA) (WILEY et al. 1981), in which monoclonal antibodies with or without neutralizing capacity served as an indispensable tool. Crystallographic studies of the three-dimensional structure of proteins serve as an additional source of information in regard to antigenic structure. For most proteins, however, although the primary structure is known, crystallographic data are not yet available. Hence, various methods based on computational analysis of hydrophilicity (ROSE 1978), high antigenicity (HOPP and WOODS 1981), or flexibility (KARPLUS and SCHULTZ 1985) of various regions in the molecule have been suggested for the prediction of the more likely antigenic determinants. Such cumulative information on segments in protein molecules with the right "ingredients" might be suggestive as regards the design of synthetic peptides that will serve as immunogens.

A completely novel and original approach for identifying epitopes that cross-react with particular antigens was recently suggested by GEYSSEN and colleagues. The strategy they employed for identifying sequential determinants in a protein involves a systematic synthesis of all its possible heptapeptide units and measurement of their reactivity with antibodies against the native protein. The peptides showing the highest reactivity presumably contain sequential epitopes (GEYSSEN et al. 1984). Alternatively, if the starting point is a monoclonal antibody that is highly reactive with the protein, it is possible to identify by systematic synthesis of all possible short peptides the one with the highest binding properties. Such

a peptide may bear little or even no direct relation to the original epitope but it mimics its ability to bind specifically to the antibody and is called a "mimetope" (GEYSSEN et al. 1985).

3 Protein-Specific Synthetic Peptide Antigens

Once antigenic determinants in a protein have been identified, it is possible to synthesize them chemically for investigation of their immunological properties. This approach has now been employed in the case of many protein antigens and has clearly demonstrated that synthetic peptides can induce antibodies that react with the intact protein in its native form. In several cases the spatial conformation was shown to play an important role in this activity, as for example in the case of hen egg white lysozyme. A fragment of this protein, consisting of residues 60–83 with a single disulfide bond and called a "loop", was shown to be immunologically active. This region, which is exposed on the surface of the molecule, is a conformation-dependent antigenic determinant (MARON et al. 1971; PECHT et al. 1971). A conjugate of a synthetic peptide corresponding to this sequence elicited antibodies with identical specificity that were reactive with native lysozyme and still recognized a conformational determinant (ARNON et al. 1971).

Another example is the coat protein of MS-2 coliphage. A CNBr fragment of the molecule P_2, consisting of residues 88–108, was identified as an antigenic region which was capable of inhibiting the process of neutralization of the phage by antiphage antibodies. A conjugate, P_2-A–L, containing a synthetic peptide corresponding to the same sequence, induced antibodies reactive with the intact protein and capable of neutralizing the viability of the MS-2 phage (LANGBEHEIM et al. 1976). Furthermore, when the synthetic adjuvant MDP (CHEDID et al. 1978) was covalently linked to P_2-A–L, the resulting conjugate elicited in rabbits very efficient MS-2–neutralizing antibodies even when administered in physiological medium, thus providing a completely synthetic antigen with built-in adjuvanticity that leads to antiviral response (ARNON et al. 1980). These findings paved the way to the study of synthetic peptide vaccines. In our laboratory the efforts in this direction concentrated mainly on two systems – influenza virus and cholera toxin (CT) – as will be described herein.

4 Synthetic Peptides Leading to Anti-Influenza Immune Response

The influenza virus provided a suitable model for studying the synthetic approach to vaccination, since detailed information is available on its structure and function, as well as its genetic variations and the serological specificities of its various strains. Influenza hemagglutinin (HA) is the major antigenic protein against which neutralizing antibodies are directed, and it is also responsible for the attachment of the virus to cells (LAVER and VALENTINE 1969). Both

the amino acid sequence (LAVER et al. 1981) and the three-dimensional structure (WILSON et al. 1981) of this protein are known, and data on its immunochemical properties (JACKSON et al. 1979) and the location of its antigenic sites (WILEY et al. 1981) are also available. Another advantage of this system is that once immunogenic fragments of it have been synthesized, the immune response they elicit can be assessed on four different levels: (a) The immunochemical reactivity, namely, the capacity of the antibodies to cross-react with the entire protein; (b) their inhibitory effect on HA biological activity; (c) the in vitro neutralization of virus plaque formation in tissue-cultured cell monolayers; and (d) the in vivo protection of mice against challenge infection.

The first peptide we synthesized, before the three-dimensional structure was known, consisted of 18 amino acid residues corresponding to the sequence 91–108 of the influenza HA molecule. This region, which is common to at least 12 H3 strains, is part of a larger cyanogen bromide fragment, previously shown by JACKSON and his colleagues (1979) to be immunologically active. According to our computer-predicted folded structure of the HA polypeptide chain, this peptide segment should have comprised a folded region with a short alpha-helical section, and hence an exposed area in the molecular structure. It also contains three tyrosine and two proline residues that had been demonstrated in our previous studies to play a dominant role in the antigenicity of several proteins or synthetic antigens. The 91–108 peptide that contains these "ingredients" was anticipated to be immunologically reactive.

Indeed, a conjugate of this peptide with tetanus toxoid elicited in both rabbits and mice antibodies that reacted immunochemically with the synthetic peptide, as well as with the intact influenza virus of several strains of type A. These antibodies were capable of inhibiting the capacity of the HA of the relevant strains to agglutinate chicken red blood cells. They also interfered with the in vitro growth of the virus in tissue culture, causing a reduction of up to 60% in viral plaque formation. But, most importantly, as shown in Table 1, mice immunized with the peptide-toxoid conjugate were partially protected against further challenge infection with the virus (MULLER et al. 1982).

Table 1. Protection of mice against challenge infection with influenza virus

Infectious strain	Group	Incidence of Infection at 10^{-2} Dilution into egg	Lung virus titer (EID^c)
A/Tex/77	Immunized[a]	19/36 (52%)	$10^{-1.98}$
	Control[b]	21/23 (91%)	$10^{-3.56}$
A/PC/75	Immunized	4/18 (22%)	$10^{-0.61}$
	Control	8/19 (42%)	$10^{-1.53}$
A/Eng/42/72	Immunized	8/18 (44%)	$10^{-1.61}$
	Control	15/21 (71%)	$10^{-2.9}$
A/PR/34(H1)	Immunized	18/19 (95%)	$10^{-3.47}$
	Control	20/21 (95%)	$10^{-3.95}$

[a] Immunized with a conjugate of the peptide 91-108 and tetanus toxoid in FCA
[b] Control groups were injected with the tetanus toxoid alone in FCA
[c] Egg infective dose

One of the crucial factors concerning the influenza vaccine is the tremendous genetic variations among the various virus strains – shifts and drifts – and their reflection on the serological specificities (LAVER and AIR 1979). The four proposed antigenic sites of the HA constitute variable regions in the molecule (WILSON et al. 1981). The peptide 91-108 was deliberately chosen to be part of a conserved region, and hence the protective effect it elicits is manifested with several strains of type A influenza virus (Table 1). This finding may serve as an indication that the synthetic approach might lead to multivalent vaccines for cross-strain protection.

Incidentally, tyrosine 98 is one of the residues that form the tentative receptor site of the molecule (WILSON et al. 1981). Although not designated by the authors as one of the antigenic sites, the peptide 91-108 could readily be visualized as an exposed region that has an immunological imprint, particularly in the infective form of the virus. This corroborates the explanation for the protective effect it elicits. It is even possible that in the intact virus this region forms a hidden determinant, but when present on a synthetic antigen, it might lead to the formation of antibodies that can reach the antigenic zone in situ and inactivate the infective virus.

As is evident from the three-dimensional structure of the influenza HA (WILEY et al. 1981), the region 140-146, which forms antigenic site A, is a "loop" of seven amino acid residues unusually protruding from the surface of the molecule. It is also exposed in the trimeric structure assumed by the HA in the spikes on the virus surface. One would expect that peptides corresponding to this region where "natural" immunogenic determinants of the HA molecule are located would elicit a better and more specific immune response against the virus. It was of interest, therefore, to synthesize peptides covering the loop region for immunization purposes. JACKSON et al. (1982) used a synthetic peptide representing the sequence 123-151, which includes the loop region, for immunization of rabbits. They did not find significant binding between the anti-peptide antibodies and the X-31 virus. However, antibodies raised against the intact X-31 virus did exhibit some binding to the synthetic peptide.

In our studies (SHAPIRA et al. 1984), four synthetic peptides were examined. Two of them corresponded to the sequence 139-146, with either Gly or Asp at position 144. The third peptide corresponded to the sequence 147-164, and the fourth included both regions and corresponded to the sequence 138-164. All four peptides, conjugated to tetanus toxoid, elicited high antibody titer against the respective homologous peptides, with a significant degree of cross-reactivity among them. Of particular interest was the high cross-reactivity between the two octapeptides differing at position 144 (Gly to Asp exchange). This finding contrasts with the significant effect of the same exchange on the serological specificity of the intact HA (LAVER et al. 1981), which is manifested in the existence of monoclonal antibody-selected variants that escape neutralization owing to this single amino acid substitution.

The four synthetic peptides differed in their cross-reactivity with the intact H3 influenza virus. Thus, antibodies induced by the two longer peptides, 147-164 and 138-164, showed significant binding with the intact virus, whereas the extent of binding of the antisera to the two short octapeptides 139-146 was essentially

insignificant. In contrast, antibodies raised against the intact virus A/Mem/102/72 or against the isolated HA were capable of recognizing the synthetic "loop" octapeptides but did not react at all with the region 147-164.

The role of the size of the peptide fragment in this system was emphasized even more in the interference of the antibodies with the biological activity of the virus, in which only the longer fragment 138-164 proved effective. Its reactivity was manifested both in the capacity of the antiserum to inhibit the HA activity of the intact virus and in the fact that active immunization of mice resulted in their partial protection against infection with the A/Eng/42/72 strain, with which the sequence of the synthetic peptide corresponds.

These results indicate that the loop region 140-146, although constituting a major naturally occurring antigenic determinant of the intact virus, is too short to fold into a loop, but that, when forming a part of the longer peptide 138-164, folding to the right conformation is facilitated. These findings are illustrative of the importance of both the length and the conformation of a synthetic peptide for its immunological reactivity.

5 CT and Coli Toxin

Detoxified bacterial toxins or toxoids have been used for vaccination, e.g., against tetanus and diphtheria, for almost a hundred years. Hence, this family of proteins seems of value as a model for investigating the synthetic approach for vaccination. Indeed, AUDIBERT et al. (1981) have shown that active immunization against diphtheria can be achieved by a synthetic tetradecapeptide consisting of residues 188-201 in the amino acid sequence of the diphtheria toxin. Conjugates of this peptide, or the hexadecapeptide 186-201, linked covalently to a protein carrier or a synthetic copolymer, elicited antibodies in guinea pigs that not only bind specifically to the toxin but also neutralize its dermonecrotic activity and lethal effect. The same results were achieved if the conjugate contained in addition the synthetic adjuvant muranyl dipeptide attached to the carrier to replace the regularly employed Freund's adjuvant. In this case a completely synthetic soluble molecule elicited antitoxin activity (AUDIBERT et al. 1982).

We have used the synthetic approach to elicit antibodies cross-reactive with cholera toxin (CT) and *E. coli* toxin that are capable of partially neutralizing their biological activity. The toxin of *Vibrio cholerae* is composed of two subunits, A and B. Subunit A activates adenylate cyclase, which triggers the biological activity, whereas subunit B is responsible for binding to cell receptors and expresses most of the immunopotent determinants. Antibodies to the B subunit are capable of neutralizing the biological activity of the intact toxin; hence, the B subunit was the obvious candidate for the pursuit of synthetic peptide immunogens. Moreover, in view of the high level of sequence homology between the B subunits of CT and the heat-labile toxin of *E. coli* (LT), we have also investigated whether the same synthetic peptides will also cross-react with *E. coli* toxin.

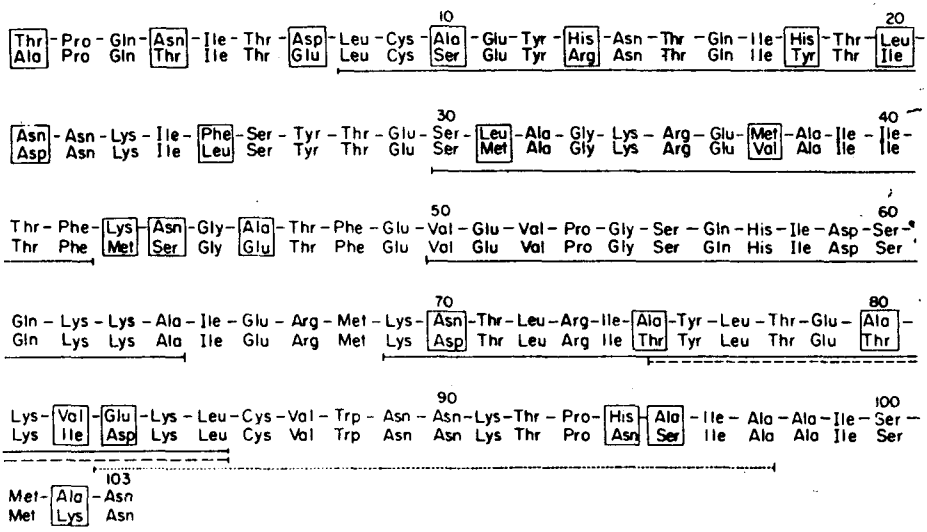

Fig. 1. Amino acid sequence of the B subunits of cholera toxin (*upper line*) and the heat-labile toxin of *E. coli* (*lower line*). Residues in the *boxes* are those that differ in the two toxins

Six peptides corresponding to sequences 8-20, 30-42, 50-64, 69-85, 75-85, and 83-97 of the CT B subunit (Fig. 1), and designated CTP 1 to CTP 6 respectively were synthesized, coupled to tetanus toxoid, and used for immunization of rabbits and mice (JACOB et al. 1983). Evaluation of the immunological reactivity of the resultant antisera by radioimmunoassay, immunoprecipitation and immunoblotting, indicated that each of the antisera reacted with the respective homologous peptide, but four antisera out of the six also reacted, to different extents, with the intact B subunit and the native CT. The antisera against the peptides CTP 1 and CTP 6 showed a very high level of reactivity with the respective homologous peptides but reacted only slightly (several order of magnitude difference) with the intact B subunit of CT. Incidentally, anti-CTP 1 also reacted with the A subunit of CT. On the other hand, peptide CTP 3 induced antibodies which, though of lower absolute titer, gave a very strong cross-reactivity with the intact toxin, very similar in its level to that of the homologous peptide-antipeptide reaction. Furthermore, this peptide was the only one that reacted with antiserum against the native CT.

Antibodies to CTP 2 (12 residues) and CTP 5 (11 residues) were reactive with the respective homologous peptides but did not react at all with the intact protein. Elongation of each of them by four or five amino acid residues resulted in peptides that induced antibodies cross-reactive with the intact B subunit and the holotoxin. However, elongation of a peptide does not always result in augmentation of reactivity; thus, for example, elongation of CTP 3 (15 residues) by 5 amino acid residues led to a lowering of the cross-reactive capacity of the elicited antibodies (JACOB et al. 1986). These findings indicate that the capacity of a peptide to transconform to fit the structure of the native protein

is dependent on its size and may be prevented either by the peptide segment's being too short to possess the correct conformation or too long and already possessing a stabilized conformation which is different from that of the protein.

Of most interest among these peptides were CTP 1 (residues 8-20) and CTP 3 (residues 50-64). Antisera against these two peptides exerted significant inhibition of the biological activity of CT. The toxic effect of CT can be demonstrated by skin vascular permeation and fluid accumulation in ligated small intestinal loops, as well as at the biochemical level, by the induction of adenylate cyclase. The inhibitory effect of the antipeptide sera was manifested in all the assays of the biological activity of the toxin with very good correlation between the biochemical level and the end biological effect of the toxin. In both cases the inhibition reached a value of approximately 60% (JACOB et al. 1983, 1984a).

As mentioned above and also demonstrated in Fig. 1, there is a high level of sequence homology between the B subunits of CT and the heat-labile toxin of *E. coli* (LT). Moreover, an immunological relationship was demonstrated between the two toxins, with the existence of both shared and specific antigenic determinants (LINDHOM et al. 1983). Since the toxin of pathogenic strains of *E. coli* is the causative agent of diarrhea in many tropical countries and, owing to its wide spread, probably presents a more serious health problem than cholera, it was of interest to investigate whether the synthetic peptides derived from CT might cross-react with the LT and/or provide a comparable degree of protection against the heterologous toxin.

Indeed, we have demonstrated that the antiserum elicited by CTP 3 (residues 50-64) is highly cross-reactive with the LT in both radioimmunoassay and immunoblotting (JACOB et al. 1984b). This is not surprising, since in this region the sequence homology between the two toxins is complete (Fig. 1). The antiserum against CTP 1 (residues 8-20) was also cross-reactive with the two toxins, though to a much lesser extent. However, antisera to both CTP 1 and CTP 3, which are inhibitory toward CT, were found equally effective in neutralizing the biological activity of the *E. coli* LT. This was manifested by the significant inhibition of both adenylate cyclase induction and the fluid secretion into ligated ileal loops of rats. The inhibition by the anti-CTP 3 was expected in view of the high level of immunological cross-reactivity. As for the anti-CTP 1, its high efficacy in the inhibition of the biological activity, contrasting with the very low serological activity, may imply that the inhibition it confers is due to an interaction with subunit A and not necessarily with subunit B, in spite of the lack of homologous sequence in the A subunit of the toxins. The immunological relationship in this case could stem from similarities in conformation rather than sequence.

Another interesting phenomenon observed with the synthetic peptides of CT was their capacity to "prime" the experimental animal toward the intact toxin. Thus, rabbits primed by a single administration of the synthetic peptide conjugates, which as such does not lead to an immune response, and subsequently boosted by a subimmunogenic dose (1 μg) of CT, too small to give any effect on its own, demonstrated a significant level of anti-CT immune response, including toxin-neutralizing capacity (JACOB et al. 1986b). The priming effect was achieved irrespectively of whether or not the immunization with