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# Peptides as potential immunodiagnostic reagents to detect mycobacterial infections

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## Introduction

The production of antigens for use as diagnostic reagents or vaccines has typically involved growing large cultures of the pathogenic organism and then producing an antigen preparation by various chemical or enzymatic means. For example, tuberculin or PPD is commonly used as a skin-test reagent to detect tuberculosis which is caused by an infection with *Mycobacterium tuberculosis* (1). Tuberculin is usually produced as follows (2,3). A virulent strain of *M. tuberculosis* is grown in a synthetic medium and after about eight weeks of growth the culture is autoclaved and filtered. Antigens are precipitated from the cell-free filtrate by the addition of trichloroacetic acid or ammonium sulfate. These crude protein preparations are calibrated against a previously characterized «standard» preparation and are then essentially ready for use in the clinic. A similar crude antigen preparation for analyzing *M. leprae* infections has been made from sonicates of irradiated or heat-killed *M. leprae* and is called leprosin-A (3,4). This type of approach to producing immunodiagnostic reagents has several drawbacks. First, one must be able to grow large quantities of the pathogenic agent. This is an enormous problem with pathogens such as *M. leprae* which can only be grown in infected animals or cells. There are also problems associated with the physical and biological containment of such large numbers of pathogenic organisms. Second, when dealing with reagents produced from live organisms there is always the chance that the final product will be contaminated with some of the virulent pathogen. For example, several outbreaks of foot--and-mouth disease in Europe have been traced back to incompletely inactivated preparations of a killed-virus vaccine (5). Third, these crude preparations are very poorly defined, often display a great deal of lot-to-lot variation in potency and chemical composition and are difficult to standardize. Also, these mixtures of mycobacterial antigens often contain components that cross-react with antigens of other mycobacteria as well as occasionally with antigens of taxonomically unrelated bacteria, thereby reducing their diagnostic usefulness (1-3).

In the past few years, two new approaches have begun to be applied to the production of bacterial protein antigens for use as diagnostic reagents. The first approach has been to use recombinant DNA technology to isolate the gene encoding a desired target antigen and then to produce that antigen in the host cell, usually *E. coli*. Although this approach can circumvent the problems involved in growing large amounts of a pathogenic organism, one is still left with the formidable task of purifying or separating the recombinant proteins from the other antigenic material produced by the host cell. This approach will be covered by other participants in this symposium and will not be discussed further here.

A second approach involves the chemical synthesis of antigens. The techniques for chemically synthesizing carbohydrates, lipids, and peptides have been available for several

decades. Numerous workers have exploited synthetic antigens to investigate various aspects of the immune response, but it has been realized only recently that chemically synthesized peptides can elicit humoral and cell-mediated immune responses that might be useful in immunodiagnostic procedures. An advantage of chemically synthesized antigens is that they are pure preparations with a known chemical composition free of biological contamination with the target pathogen. My laboratory has recently begun to apply this technology to the study of the antigens of mycobacteria. In order to illustrate the synthetic peptide immunogen approach, I will briefly review some of the features and applications of synthetic peptide antigens and immunogens and then discuss our recent results with peptide antigens of *M. tuberculosis*. I will also discuss the possible applications of this technology to *M. leprae*.

**Synthetic peptide immunogens**

A little over twenty years ago, Anderer (6) showed that a short peptide corresponding to the amino-terminal six residues of the coat protein of the tobacco mosaic virus could elicit antibodies that bound to the peptide, the full-length coat protein, and even the intact virus. This observation was exploited by a number of workers, most notably Atassi, Sela, Anfinsen and their colleagues, to investigate the host's immune response to proteins. Unfortunately (for a synthetic peptide immunogen-based technology), their studies led to the general conclusion that in order to elicit protein reactive antibodies it was necessary to design a peptide that displayed or mimicked the tertiary structure of the corresponding region of the full-length protein. Furthermore, for a typical globular soluble protein such antigenic sites were thought to be few in number (4-6 sites per protein) and small (each site involving only 4-7 key amino acids). Therefore, given these conclusions, it seemed unlikely that the use of peptides to elicit protein-reactive antibodies would find widespread use. (For a review of these studies and the then prevailing concepts of the immunogenicity and antigenicity of proteins see references 7 and 8.)

In 1980, signals began to appear which indicated that a peptide based-immunogen technology was indeed feasible and might be widely applicable to many studies (9). Two of the studies that were instrumental in establishing the potential usefulness of peptides to elicit protein-reactive antibodies involved peptides corresponding to portions of the amino acid sequences of the hemagglutinin protein of the influenza virus (10) and the surface antigen of hepatitis B virus (11). Of 26 peptides studied, 24 elicited antibodies that could react with the intact cognate protein. More importantly, the only apparent requirement for success was that a portion of the peptide's sequence correspond to a surface-accessible region of the full-length protein. In other words, these studies indicated that the information carried within a short peptide is often sufficient to elicit antibodies reactive against a much larger protein molecule and that the approach should be successful with most regions of a protein. These studies and the current concepts of why linear peptides can elicit protein reactive antibodies have been reviewed and discussed numerous times recently (12-15) and will not be described in detail here. What is important for this discussion are the possible applications of the peptides and peptide-elicited antibodies in basic research and medicine.

**The possible uses of peptides as immunogens**

Synthetic peptide immunogens typically elicit a population of antibodies, some of which can react with the cognate protein in its native conformation and some of which can react with it in its denatured conformation. [In contrast, antibodies elicited by one conformational state of a protein rarely react with that protein when it is in another conformational state (16).] This means that peptide-elicited antibodies should be usable in almost any assay ranging from western blots and ELIZAs to enzyme inhibition studies. Indeed, in my laboratory the same

peptide-elicited antiserum has been successfully used in ELIZA's, western blots, immunocytochemical studies, immunoprecipitation under «native» and denaturing conditions, immunoaffinity purification protocols, and enzymatic activity inhibition studies.

A key feature of the peptide-elicited antibodies are that they react with a small region of the cognate protein that is chosen in advance by the investigator. The ability to direct these antibodies to predetermined sites can be used to produce reagents that can distinguish between closely related proteins. For example, the Thy-1 antigen is a 112-amino acid glycoprotein which exists in two forms that differ by a single amino acid and which are encoded by two alleles at the Thy-1 locus (17). The Thy-1.1 antigen has an arginine at postition 89, whereas the Thy-1.2 antigen has a glutamine at position 89. Alexander *et al.* (18) synthesized peptides corresponding to the two variant sequences as well as peptides corresponding to shared sequences. Antibodies elicited by peptides corresponding to the shared sequences reacted with both allotypic forms. Antibodies elicited by the peptides representing the variable sequences showed the corresponding specificity for reacting with Thy-1.1 or Thy-1.2. Therefore, Alexander *et al.* (18) were able to produce allotype specific antisera by targeting the antibodies to the regions that differed in these two closely related antigens.

Such specific antisera are relatively easily produced compared with the conventional means available to produce antibodies that can distinquish between two closely related proteins. One can imagine that the peptide-elicited antibodies that react with the different Thy-1 proteins in combination with the peptide-elicited antibodies that react with both Thy-1 proteins might form the basis of an ELIZA assay to detect and type Thy-1 antigens. Such specific reactivity might also be exploited in the serotyping of highly cross-reactive pathogens. For example, there is a large amount of cross-reactivity between the various mycobacterial species and one could imagine a series of peptide-elicited antibodies each targeted to a region of an antigen that is specific for a given mycobacterial species. Such antibodies might be useful in the rapid identification and characterization of unknown mycobacteria. The possible advantages of such peptide-based immunoassays would be the ease of production of both specific and cross-reactive antibodies directed against the same antigen, the availability of a stable, easy-to-produce positive control (the peptide), and the absence of the pathogenic agent during the production of the reagents.

As immunogens, peptides may be useful in eliciting a desired, specific immune response. Perhaps, peptides might form the basis of chemically-defined, synthetic vaccines. The possible advantages of such a vaccine would include that one does not have to grow or handle large quantities of the pathogenic agent and the corresponding absence of possible biological contamination with the pathogenic agent, the excellent stability of peptides at room temperature, and the ability to define precisely the immunogen chemically. In the laboratory, peptide-based vaccines show promise of providing immunologic protection from infection with hepatitis B virus, herpes simplex virus, foot-and-mouth disease virus, poliovirus, rabies virus, enterotoxic *Escherichia coli,* and *Plasmodium falciparum,* the malarial parasite (see ref. 19 for primary research articles and a general overview). None of these potential peptide-based vaccines have yet to reach the clinical testing stage. However, given the possible benefits of a peptide-based vaccine, work is proceeding rapidly on their development.

**Possible uses of peptides as antigens**

The peptides themselves may be useful in immunodiagnostic procedures. For example, some peptides can react with antibodies that are elicited by the intact protein or pathogen (20). Such a peptide might be useful in an ELIZA to detect the presence of antipathogen antibodies in various clinical specimens. Advantages of such an ELIZA might include the stability of the peptide antigens at room temperature, the ease of manufacture of the peptides, and the

possibility of using soluble peptide as a competitor of the reaction to demonstrate the specificity of the reaction. Alternatively, the ability of peptides to elicit cellular immune responses may be useful in developing a peptide based delayed cutaneous hypersensitivity test. To illustrate this, I describe below our studies with one particular peptide that corresponds to an antigen of *Mycobacterium tuberculosis.*

A crude mixture of mycobacterial antigens (tuberculin) is commonly used as the test reagent in delayed cutaneous hypersensitivity assays to detect *M. tuberculosis* infections. Numerous attempts have been made to fractionate the antigens present in tuberculin by various enzymatic or chemical means in an effort to identify and characterize the immunologically important components (reviewed in 3, 21, 22). The ultimate goal of these studies has been the production of a better defined, less cross-reactive skin test reagent. My laboratory has been approaching this goal through a combination of the monoclonal hybridoma, recombinant DNA, and synthetic peptide technologies. That is, we and other laboratories have been using monoclonal hybridoma antibodies to identify and analyze the mycobacterial antigenic repertoire (summarized in ref. 23 and 24). In particular, the monoclonal hybridoma antibodies are used to identify mycobacterial epitopes that are expressed specifically or predominantly by the tuberculous species. The thought being that such antigens should be good candidates for more specific skin test reagents. The monoclonal hybridoma antibodies are then used as the specific probes to identify recombinant DNA clones that express the mycobacterial antigens in *E. coli.* Such recombinant DNA-produced antigens might be usable as skin test reagents provided that they can be satisfactorially purified and separated from the *E. coli* proteins. The nucleotide sequences of the genes encoding the mycobacterial protein antigens are determined and the amino acid sequences of the proteins deduced. Then using the protein sequences as blueprints, peptides are synthesized and tested for immunologic activity. A key question in this approach is whether or not peptides can elicit useful delayed cutaneous hypersensitivity reactions in persons experiencing a mycobacterial infection. Our studies described below indicate that peptides may indeed form the basis of a more specific skin test.

We have been studying a 13-residue peptide that corresponds to the amino terminus of a 10,000 dalton protein called BCG-a (25, 26). The BCG-a protein was identified using a monoclonal antibody isolated from a mouse immunized with a sonicate extract of *M. bovis* BCG and was shown to be expressed predominantly by the tuberculous mycobacterial species although it can be found in lower amounts in some other mycobacterial species, most notably in *M. intracellulare* (25). To determine if this 13-residue peptide could elicit a potentially useful delayed cutaneous hypersensitivity reaction, we immunized guinea pigs with sonicate extracts of various mycobacteria and then assayed the ability of a 250-microgram dose of the peptide (which had been polymerized through cysteine residues attached to either end) to elicit a delayed cutaneous hypersensitivity (DCH) reaction (26). The polymerized peptide did elicit a strong DCH reaction in animals immunized with the extracts of the tuberculous mycobacteria (20/20 animals tested) and not in animals immunized with extracts of *M. fortuitum* or *M. kansasii.* Three of ten animals immunized with an extract of *M. intracellulare* also displayed a DCH reaction to the polymerized peptide. All these animals displayed a strong reaction to 5 TU of PPD. These results indicate that peptides can elicit potentially usefull skin test reactions and that peptide antigens may display much greater specificity in skin tests than tuberculin.

Considering that microgram or sub-microgram doses of tuberculin can elicit a strong DCH reaction, the 250 microgram dose of peptide that was required is quite a large amount to use. We suspect that the BCG-a peptide corresponds to a «poor» or immunorecessive T-cell immunogen or antigen when one considers the overall immune response to an invad-

ing mycobacterium and hence a large dose of peptide is required to elicit a strong reaction. What sort of peptide would be a good candidate for a clinically useful skin test antigen? Ideally, the peptide should correspond to a species-specific T-cell epitope that elicits a major response during infection with the target pathogen (i.e., an immunodominant epitope). For both *M. leprae* and *M. tuberculosis,* it appears that a large fraction of the T-cells elicited during infection are directed against a 65,000-dalton protein which has been designated the 65KD antigen (23, 24, 27-30, T. Godal and S. Kaufmann, personal communications). This protein contains B and T-cell epitopes that are shared between the 65KD antigens isolated from various mycobacteria as well as epitopes that appear to be expressed only by individual species, that is, species specific epitopes (23, 24). Therefore, one suspects that peptides corresponding to portions of the 65KD antigen might be useful immunodiagnostic reagents.

The 65KD antigens from *M. tuberculosis* (31), *M. bovis* BCG (30, J. van Embden, personal communication), and *M. leprae* (32) have been cloned and their nucleotide sequences determined. The *M. leprae* 65KD antigen contains 541 amino acids while the *M. tuberculosis* and *M. bovis* BCG 65KD antigens contain 540 amino acids. At the amino acid sequence level, The *M. tuberculosis* and *M. bovis* BCG 65KD antigens are identical while they are are about 95 % homologous to the *M. leprae* 65KD antigen (33). This is surprising in that at the nucleotide sequence level the *M. leprae* and *M. tuberculosis* genomes are only about 30 % homologous (34). The observed high degree of homology suggests that there is very strong evolutionary pressure to maintain the primary amino acid sequence of this protein. (This in turn raises the possibility that this protein might be a good candidate for a subunit vaccine against mycobacterial infections.) Richard Young and colleagues (32) have mapped the binding sites for six different monoclonals reactive with the *M. leprae* 65KD antigen. Some of these antibodies cross-react with the *M. tuberculosis* 65KD antigen whereas others do not. As expected, the cross-reactive epitopes are in regions common to the two antigens while the specific epitopes map to regions that differ in the two sequences.

Given this information, how could one go about developing a new skin test reagent. The types of peptide reagents that we would like to obtain are peptides that elicit a DCH in persons infected with either mycobacterium (i.e., a broadly cross-reactive epitope) as well as peptides that elicit a DCH only in persons infected with one or the other of these two mycobacteria. Therefore, we are synthesizing peptides that correspond to the shared sequences as well as peptides that correspond to the unique sequences of the 65KD antigen. The peptides will then be tested for their ability to elicit a T-cell proliferative response in cloned T-cells as well as in cultures of peripheral blood lymphocytes from individuals experiencing a mycobacterial infection. Peptides that show promising behavior will then be tested for their ability to elicit a delayed cutaneous hypersensitivity reaction in infected or immunized animals and humans.

**Summary**

Chemically synthesized peptides have been shown to be able to elicit immunologic reactions useful in the laboratory. These results suggest that clinically useful peptide or peptide-elicited antibody-based assay systems to detect mycobacterial antigens or anti-mycobacterial antibodies are feasible. Peptides are also excellent candidates for a new generation of skin test reagents to detect a T-cell response elicited by the infecting mycobacterium. The potential advantages of such a reagent are that it is easy to manufacture, stable at room temperature, chemically defined, and, perhaps, more specific than tuberculin for detecting a particular mycobacterial infection. What needs to be done in the immediate future is the identification of the optimal peptide by a series of *in vitro* assays of immunologic reactivity and specificity and then an analysis of its potential clinical usefulness. Two candidates to focus our current

attention on are the 65KD antigens of *M. leprae* and *M. tuberculosis*. Of a much more speculative nature is the possibility that a peptide corresponding to a portion of the highly conserved 65KD antigen that elicits a strong DCH reaction might also be a good candidate for a synthetic peptide vaccine against tuberculosis or leprosy. A great deal of work remains to be done before we will know if such a vaccine is even feasible. Nonetheless, it seems clear that the synthetic peptide immunogen and antigen approach should generate new, clinically useful reagents for the immunodiagnosis of tuberculosis and leprosy.

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