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The Leeuwenhoek Lecture, 1993: Peptide Vaccines: Dream or Reality?

Fred Brown

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The Leeuwenhoek Lecture, 1993. Peptide vaccines: dream or reality?

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SUMMARY

Small fragments of micro-organisms which elicit protective immune responses have now been identified for several disease-causing agents. This major advance has made it possible to envisage the chemical synthesis of vaccines which could replace those in current use and may also furnish products which cannot be made by traditional methods. In my lecture I will illustrate the principles involved by describing the advances made with synthetic vaccines for foot-and-mouth disease, hepatitis B and malaria.

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1. INTRODUCTION

The Royal Society's Leeuwenhoek Lecture was established as the result of a bequest made in 1948 by Mr George Gabb for an annual lecture in the field of microbiology. Not only is it a privilege to be invited to give this lecture but the task of preparing it has given me considerable pleasure because it stimulated me to read again about this very unusual Dutch pioneer in microbiology. In fact, Leeuwenhoek was the first of the microbe hunters, so vividly described in Paul de Kruif's '*Microbe hunters*' (de Kruif 1926). He was also my first microbe hunter because, like many other colleagues, I was introduced to microbiology by reading de Kruif's book. Coincidentally I first read the book in the year in which the George Gabb Fund was established.

Reading this book was instrumental in shaping my career because I decided to apply my chemical training to a study of microbes, and in particular viruses. In Leeuwenhoek's time, shortly after the foundation of the Royal Society, there was no firm concept about the cause of infectious diseases and his observations led to the discovery of a whole new world. As de Kruif (1962) has written 'Caesar had gone to England and come upon savages that opened his eyes with wonder – but these Britons were as ordinary to each other as Roman centurions were to Caesar; huge elephants amazing to Alexander the Great but commonplace to Hindus; the Pacific Ocean astounding Balboa but ordinary to Central American Indians. But Leeuwenhoek? This janitor of Delft had stolen upon and peeped into a fantastic sub-visible world of little things . . .; beasts these were of a kind that ravaged and annihilated whole races of men ten million times larger than they were themselves'.

The subject of my lecture is vaccination. But before embarking on this topic, I would like to quote parts of an editorial which appeared recently in the journal *Science* (Koshland 1992). 'In the 1930s, a visitor to a

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hospital would have found the wards filled with victims of infectious diseases such as pneumonia, tuberculosis, typhoid fever and encephalitis. In 1985, the visitor would have found many patients suffering from organic diseases such as cancer, heart disease and strokes rather than from an infectious disease. This dramatic shift led some officials (including some in our own Medical Research Council) to proclaim in the 1980s that the era of microbial diseases as a threat of mankind had passed. But declaring a triumph over the wily bacterium or its henchman the virus was premature. The parasites, bacteria and viruses have mutated'.

My presentation will be partly historical, but most of what I have to say will deal with what I see as the future of vaccination. I will start by tracing the way in which the practice has developed since the early beginnings in India in the 10th Century, its spread to China, the Middle East and then to England early in the 18th Century, followed by the experiments of Benjamin Jesty and Edward Jenner (whose paper on vaccination was rejected by the Royal Society) which really saw the beginning of vaccination as we know it today. It was almost a century later that the much neglected Greenfield (1880) described a vaccine against anthrax, Pasteur provided vaccines against chicken cholera (Pasteur 1880), anthrax (Pasteur *et al.* 1885) and rabies (Pasteur 1885), and Pasteur and Koch and their colleagues founded the new science of microbiology.

The publicity surrounding the demonstration by Pasteur of his anthrax vaccine at Pouilly-le-Fort near Paris and the post-exposure protection of Joseph Meister and Jean Baptiste Jupille, both of whom had been bitten by rabid dogs in France, ignited the interest of those responsible for public health. The agents causing diphtheria, tetanus, tuberculosis and the plague were isolated in rapid succession and efforts were made to produce vaccines against them. Of particular significance was the demonstration that multiplication of the causal agent was not necessary in order to achieve protection. Although there was confusion in the identification of the organism causing hog cholera, nevertheless Salmon & Smith (1886) demonstrated that heat inactivated cultures of Salmonella cholerae suis would protect experimental animals against infection.

But probably of greater importance, as we move towards vaccines based on sub-units of micro-organisms, was the demonstration that the secreted toxins of the organisms causing diphtheria and tetanus elicited antibodies which, when passively transferred, protected patients suffering from these diseases (Behring & Kitasato 1890). Several years later, Ramon (1923) in France and Glenny & Hopkins (1923) in England showed that active immunization against the diseases could be evoked by inoculation of the de-toxified toxins. These were the first sub-unit vaccines.

Building on these foundations we have now reached the stage where we can make classical vaccines against several important diseases for both man and his domesticated and pet animals. These vaccines are not perfect but, apart from the provision of clean water, they represent our best, and cheapest, defence against many diseases. Many regard them as the most cost-effective measure in both human and veterinary medicine. The big breakthrough in the production of viral vaccines came with the discovery of antibiotics which allowed large-scale culture of the agents outside the animal body. Since the discovery of antibiotics in the 1930s and 1940s, however, vaccination against bacterial diseases has been somewhat neglected, but nevertheless vaccines still have an important role in the control of diseases such as diphtheria, tetanus, tuberculosis and whooping cough.

However, the advances made in the past 100 years have been largely technical and the same principles discovered and applied by Jenner, Pasteur and Theobald Smith are still being used. Killed vaccines are produced by inactivating the wild-type organism under conditions which do not impair its immunogenic activity. Live attenuated vaccines are produced from naturally occurring strains (e.g. smallpox and Marek's disease) or from strains which have been selected or weakened by several passages in an unnatural host (e.g. yellow fever) or tissue culture cells. Even the idea of sub-units is not new because the toxins excreted by growing the agents of diphtheria and tetanus in the test tube, suitably inactivated or toxoided, have been in widespread use for many decades.

Despite some shortcomings, the classical vaccines in use today have been highly successful. We have seen the eradication of smallpox, and viral diseases such as measles, mumps, poliomyelitis, rubella and yellow fever rarely occur in developed countries. Similar success has been achieved with bacterial diseases such as diphtheria, tetanus, tuberculosis and whooping cough. There are equally successful vaccines being used in veterinary medicine, with products that control leptospirosis, rinderpest and foot-and-mouth disease in cattle, clostridial diseases in sheep, diarrhoea caused by enterotoxigenic *E. coli* in pigs, and Newcastle disease and Marek's disease in poultry.

The classical approach has been so successful that it is fair to ask why we should even consider other approaches. There are several reasons.

1. Even the successful vaccines can cause undesirable side reactions, as perceived in whooping cough vaccination and clearly demonstrated with the rabies vaccines prepared from brain tissue.

2. There is a hazard to personnel working with large amounts of human pathogens (e.g. rabies virus) and to the environment when working with an agent which will infect livestock (e.g. foot-and-mouth disease virus).

3. Attenuated strains can always revert to virulence.

4. With killed vaccines, there is the need to ensure complete inactivation.

5. Refrigerator temperatures are required for storage and transport.

6. There are some diseases for which the classical approach is not possible at present because the agent cannot be grown in sufficient quantities. An example of this is hepatitis B virus. Encouragingly, another

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route has been found for this disease and there is a highly successful vaccine available.

2. NEW VACCINES

A major task in developing new vaccines has been to identify in complex molecules such as bacteria, parasites and viruses, those functional units which elicit protective immunity, and whose structures are amenable to high resolution analysis. A second and more demanding task is to produce those units and present them to the host in a functional configuration. A fact which is often overlooked by those sceptical of this approach is that, as mentioned above, two of our highly successful vaccines, those against diphtheria and tetanus, are in fact sub-unit vaccines.

Dissection of micro-organisms into biologically functional sub-units started in the 1960s as the molecular approach to biology began to make its impact on bacteriology and virology. Because of their simpler structures, the early inroads were made with viruses. It was shown that viruses such as those causing influenza, measles, rabies or vesicular stomatitis, all of which have a lipid coat, could be dissected into sub-units which had biological activity (reviewed by Brown 1984). In particular, the surface projections of these viruses were shown to induce neutralizing antibody. With the exception of the haemagglutinin of influenza virus, these sub-units have not been used as vaccines except in experimental animals. One reason is that, compared with the intact organism, the level of protective antibody induced is much less, unless the method of presentation is correct. However, the work of Morein and his colleagues with the ISCOM (*I*mmuno *S*timulating *COM*plex) method of presenting proteins as cage-like structures, which resemble spherical virus particles, has gone a long way towards solving this problem (Morein *et al.* 1990).

Consequently, when methods were developed for expressing genes in bacterial and viral vectors, there was already considerable information on proteins which can evoke protective immune responses. Two main routes are available: (i) expression in a bacterium such as *E. coli* or *B. subtilis*, in yeast (*Saccharomyces cerevisiae*) or in a baculovirus such as *Autographa californica* (Matsuura *et al.* 1987) to produce antigens which can be formulated as inactive vaccines (reviewed by Brown 1984); or (ii) presentation in an already accepted live vaccine such as vaccinia virus (Mackett 1990), adenovirus (Graham & Prevec 1992) or BCG (Bloom *et al.* 1990).

So far, vectors have been used in a logical but naive way for delivering foreign antigens to the immune system, but it is now realized that it is not sufficient simply to produce large amounts of the required antigen. *In vitro* expression of immunogenic proteins as a method for producing new vaccines has been disappointing because it was not appreciated by molecular biologists that a protein isolated from a micro-organism is rarely as immunogenic as the same protein when it is a constituent of the parent organism. This activity, which can be as low as 1% of that of the same protein *in situ*, is almost certainly due to the alteration in configuration when it is released from the constraints imposed by the architecture of the parent organism. Presentation of the expressed protein in a configuration identical with, or at least similar to, that which it has on the parent organism would seem to be a pre-requisite for the retention of its immunogenic properties.

These considerations were neglected by the molecular biologists who first invaded the field of vaccination. It was almost as if Ehrlich and Landsteiner had never lived. Maybe they had never heard of them.

So far, all that has been achieved by the methods I have referred to is the expression of proteins in a foreign milieu. Although such an approach is remarkably sophisticated biochemically, it is entirely fortuitous if an immunogen produced in this way is effective, presumably because it folds in the same way as it does in the parent organism. But it does not provide any clues about how to achieve the immune responses we seek from rationally designed vaccines. These clues can only be obtained by dissection of the proteins into active peptide fragments.

3. PEPTIDES AS VACCINES

There are several reasons for the interest being shown in the potential of synthetic peptides as vaccines. Chemical synthesis allows the production of stable products which are not bedevilled by the problems associated with materials produced by biological procedures. Moreover, the ease with which they can be synthesized, compared with even five years ago, has made this approach more feasible. Peptide vaccines also offer the advantage that their simple structure, compared with that of proteins, allows them to be manipulated more readily. Probably of more importance, however, is the fact that as we learn more about the immune response at the molecular level, it becomes increasingly obvious that the interactions of the MHC molecules with proteins involves only short amino acid sequences.

(*a*) *Conceptual ideas*

The earlier conceptual opposition to the idea that peptides could be used as vaccines stemmed from the perception that the sites on proteins which were recognized by B cells consisted of amino acid sequences situated near each other in the native protein but distantly in the primary sequence.

Another distinction between peptides and proteins as antigens is not that the former are smaller than the latter but that a protein exists in a single or very small number of tertiary structures whereas a peptide in solution exists in a large number of different configurations. Consequently it was argued that a peptide would not necessarily elicit the same response as that elicited by the corresponding region of the protein of which it forms a part. Nevertheless, Anderer and Sela and their colleagues had shown many years ago that antibodies which reacted with and neutralized tobacco mosaic virus and the bacteriophage MS2 could be elicited by inoculation of peptides corres-

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ponding to linear sequences in the respective viruses (Anderer 1963; Langebeheim, Arnon & Sela 1976). This meant that the two viruses possessed linear determinants of biological relevance. With the description of methods for the sequencing of nucleic acids in the late 1970s, the sequences of many genes coding for proteins of immunological interest have become available. The basic problem is to identify the sequences of amino acids which evoke a protective immune response.

(*b*) *Location of immunogenic epitopes*

The direct approach of hydrolysing the immunogenic protein with enzymes or reagents which cleave at specific amino acids (e.g. cyanogen bromide at methionine residues) and testing the fragments for activity was the first method to be used (Anderer 1963; Langebeheim *et al.* 1976). In an indirect approach, Bittle *et al.* (1982) reasoned that the antigenic variation which is an important feature of foot-and-mouth disease virus would be reflected in amino acid sequence variation in the immunogenic protein. This method proved to be successful because comparison of the derived amino acid sequences of viruses belonging to three different serotypes pinpointed these regions of variability, one of which coincided with a tract on one of the proteins which is cleaved by trypsin, an enzyme which reduces the immunogenicity of the intact virus by at least two orders of magnitude. A peptide corresponding to this sequence elicited levels of neutralizing antibody which protected experimental animals against challenge infection.

A method which has been found to be extremely valuable in the identification of linear epitopes was described by Geysen and his colleagues (Geysen *et al.* 1984). In this method overlapping hexapeptides comprising amino acids 1–6, 2–7, 3–8, . . . etc. of the entire protein are synthesized on polyethylene rods and then allowed to react with neutralizing antiserum. Those peptides to which antibody attaches are detected with anti-species antibody. This approach has been extended into a more sophisticated method in which combinatorial libraries of peptides are produced and the peptide reacting with the specific antibody is sequenced. This allows epitopes on proteins to be identified without the need to know the sequence of the protein (Lam *et al.* 1991; Houghten *et al.* 1990).

Several predictive methods have also been described, using the primary sequence of the protein or properties that are related to folding. All the models assume that antigenicity is a surface property and that accessibility on the surface of the native protein is a minimum requirement. Properties such as hydrophilicity, the occurrence of β-turns, protruding regions, segmental mobility and the presence of specific amino acids have all been used.

In all the initial experiments the sequence of amino acids predicted to react with the native protein or micro-organism was coupled to a protein molecule such as keyhole limpet haemocyanin or bovine serum albumin before injection into the host. This step was considered to be necessary because it was thought that peptides would behave like haptens and would not be immunogenic unless they were coupled to a protein. However, in our own work with foot-and-mouth disease virus, we found (Francis *et al.* 1987*a*) that a peptide corresponding to what has subsequently been shown by X-ray crystallography to be an exposed loop region on one of the capsid proteins (Acharya *et al.* 1989) elicited neutralizing antibody in guinea pigs without being coupled to a carrier protein. Unfortunately the response of other species, such as cattle and pigs, to the uncoupled peptide was disappointingly low. These observations, however, focused attention on the need to include T-cell recognition sites in the design of synthetic vaccines. Such a requirement emerged from studies of the immune response at the molecular level.

(*c*) *Importance of T-cell epitopes*

Vaccines function by influencing the adaptive immune response. The immunoglobulin receptors on B lymphocytes recognize the three-dimensional structure of the antigen, thus conferring the specificity of the immune response. The antigen is phagocytosed by macrophages and degraded to peptides, some of which bind to class II major histocompatibility antigens and are displayed on the cell surface. This complex is recognized by the receptors on T cells, which differentiate and proliferate to become effector cells which mediate help or delayed-type hypersensitivity.

Consequently a vaccine must contain, in addition to a B-cell epitope which confers specificity, a T-cell epitope suitable for the host species, since this is class II MHC restricted. Because T cells recognize processed fragments of protein antigens, configuration of the peptide is not regarded as a serious problem for this facet of the immune response. However, there is still insufficient information available to allow conclusions to be reached about the relevance of the relative positions of the B cell and T cell epitopes on a protein to the immune response. There are several examples in which added T cell epitopes have been shown to overcome genetic resistance to the response elicited (Borras-Cuestas *et al.* 1987; Francis *et al.* 1987*b*; Good *et al.* 1987; Leclerc *et al.* 1987).

In any case the T cell response appears to be focused on a limited number of determinants. The basic problem is to identify T cell epitopes that are suitable for a particular species. With animals whose genetic background is well defined this is not too large a task. However, when there has been extensive cross breeding, as in most human populations, the problem is greater. Nevertheless, T cell epitopes could be selected from an antigen such as tetanus toxin for which the response in most individuals is good.

4. FOOT-AND-MOUTH DISEASE, HEPATITIS B AND MALARIA

I now wish to discuss our state of knowledge with the peptide approach to vaccination against three diseases. Key B cell epitopes on foot-and-mouth disease virus, hepatitis B virus and the malaria parasite have

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been identified and tested in experimental animals. The major epitope on foot-and-mouth disease virus is located on a flexible loop region and antibodies produced against a peptide corresponding to this loop neutralize the infectivity of the virus. Moreover, more than 30% of these anti-peptide antibodies react with the virus particle. Many experiments by several groups have shown clearly that experimental animals not only elicit high levels of neutralizing antibody when injected with peptides corresponding to the highly immunogenic loop of the virus, but they are also protected against challenge infection with high doses of infectious virus. The experiments with cattle and pigs have been less conclusive but there is little doubt that protection can be obtained with peptides (reviewed in Brown 1992).

In hepatitis B virus, the surface antigen has four serologically defined sub-types, adw, ayw, adv and ayr but protection against infection is conferred by antibodies to the group-specific determinant. The location of this determinant and its precise definition still remain to be established but peptides encompassing the 110–150 region of the surface protein elicit antibodies that react with the antigen. As with the foot-and-mouth disease virus peptide, presentation of the hepatitis B peptide is important, particularly with respect to the influence which the cysteine residues have on its conformation. Reduction of the disulphide bonds followed by alkylation results in the loss of most of the immunogenicity of the peptide.

In recent work, Thornton *et al.* (1989) have described the protection of chimpanzees from hepatitis B infection by inoculation of a peptide from the pre-S region. Although much remains to be done, it is clear that protection is possible by inoculation of an appropriate peptide.

The development of a peptide vaccine for malaria presents an even greater challenge because of the complexity of the life cycle of the parasite, the structure of the parasite itself and its antigenic variability. However, the fact that man can be protected against infection with *Plasmodium falciparum* by vaccination with whole malaria parasites has persuaded several groups to investigate the possibility of producing a sub-unit or peptide vaccine. Most attention has been concentrated on sporozoites because these, after inactivation, had been shown to protect fowl against infection with *Plasmodium gallinaceum*. Anti-sporozoite antibodies have neutralizing activity and a monoclonal antibody against *P. berghei* sporozoites had all the properties of polyvalent sera from infected animals. Decisively, passive transfer of the monoclonal antibody afforded protection against challenge. By using this monoclonal antibody, a surface protein of M_r 4×10^3 was identified as being important in evoking protection.

The genes coding for this protein in *P. knowlesi*, *P. falciparum*, *P. vivax* and *P. cynomologi* have a central region, containing tandem repeat sequences, which is the main target of the immune response. In *P. falciparum* the repetitive epitope consists of the tetramer Asn-Ala-Asn-Pro which is identical in sporozoites from widely separated geographical areas. Sequential repeats of the tetramer induce anti-sporozoite activity and in a small trial in humans there was some evidence that this activity afforded protection.

During the past few years Patarroyo and his colleagues (Patarroyo *et al.* 1988; Amador *et al.* 1992) have constructed a peptide SPf66 which is based on sequences from three merozoite proteins and the repeat sequence Asn-Ala-Asn-Pro from the sporozoite protein rather than on a single sub-unit. This approach, although to some extent empirical, seems to be effective. In well controlled field trials, the protective efficacy was as high as 77% in children aged 1–4 years and 67% in adults older than 45 (Valero *et al.* 1993). These results show clearly that the peptide approach to vaccination should not be neglected by those seeking new products.

In all three diseases, T cell epitopes have been identified which provide help (Borras-Cuestas *et al.* 1987; Francis *et al.* 1987*b*; Leclerc *et al.* 1987; Good *et al.* 1987). Moreover, the demonstration by Milich *et al.* (1986) that the hepatitis B core protein provides considerable help to peptides of the surface antigen has been shown to be very significant. In independent experiments my colleagues have found that linking of the foot-and-mouth disease virus peptide to the hepatitis B virus core enhanced its immunogenicity spectacularly (Francis *et al.* 1987*b*) and they have shown subsequently that this approach also works well with other peptides (Brown *et al.* 1991).

Probably the most significant information regarding the problem of genetic restriction of the antibody response has been the demonstration by Sinigaglia *et al.* (1988) that T cell epitopes can function in a wide range of MHC backgrounds. This would reduce the number of T cell epitopes required to provide help for antibody production in a high proportion of recipients. Indeed the search for a generally applicable T cell epitope probably needs to go no further than tetanus toxin.

5. PRACTICAL IMPLICATIONS

In a subject as applied as vaccination, the question will always be whether such an approach will ever be commercially feasible. Arguments about scientific advantages may be accepted by scientists but they are unlikely to convince accountants. When a product against a particular disease already exists, any new product, whether it is a peptide, a recombinant protein or a newly created attenuated vaccine, will only be accepted if it is better or cheaper or preferably both. In those situations where there is no available product, these considerations do not apply. Moreover, accountants do not view kindly efforts to make vaccines for Third World diseases. Consequently, most research on these diseases has been done in academic and Government laboratories.

Nevertheless it is worthwhile to spell out the scientific and clinical advantages of a peptide vaccine (table 1). Of particular importance in the list shown in the table is the fact that a chemically defined product would be used rather than the soups we still use today. A second advantage would be the opportunity a

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Table 1. *Advantages of a peptide vaccine*

1. Product chemically defined
2. Stable indefinitely
3. Unlimited supply
4. No infectious agent present
5. No large-scale production plant required
6. No downstream processing required
7. Can be designed to stimulate appropriate immune responses by incorporating both B and T cell epitopes
8. Provides opportunity to use delayed release mechanisms
9. No cold chain required

peptide vaccine provides to use a delayed release mechanism. At present, two or more injections of a vaccine are usually given, provided the recipient attends for the second and subsequent injections. Systems have now been devised which allow triggered release at different intervals, allowing booster doses to be provided without injection. Our own experiments with the foot-and-mouth disease virus peptide have shown that its immunogenic activity is unimpaired by the required processing or by its sojourn in the body before release. Such an advance in the presentation of a vaccine would have a significant impact on vaccination procedures.

6. CONCLUSIONS

In any discussion on the potential of peptides as vaccines, it is useful to identify those immune responses which are obtained with the current successful products and to determine whether the same responses can be elicited with peptides. Because a peptide, however complex, is only representative of what is present in the entire organism, it is somewhat bewildering to the author that the 'peptide approach' has met with such pessimism, particularly in this molecular age when an increasing number of biological phenomena can be explained in molecular terms. Study of the immune response has now left the era of the 'black box' and entered the molecular age. Consequently we can now make some rationally based guesses on what we should try to make.

In our present state of knowledge there are two clear objectives: the peptide should contain (i) the epitope(s) important for protective antibody production and (ii) T cell recognition sites that induce antibody production and cellular immunity and prime memory cells that will provide long-term immunity. There is much talk about drug design. Can we not approach vaccine design in the same way? Merely expressing immunogenic proteins teaches us very little about what is required for designing successful vaccines.

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