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The Impact of New Technologies on Vaccine Development

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Introduction

Vaccination has proved to be an extremely effective approach to protecting human and animal populations from the ravages of infectious diseases caused by micro-organisms. Immunoprophylaxis is a highly attractive option because vaccination can promote long-lasting immunity against many infectious diseases in the absence of debilitating clinical symptoms, and the spread of potentially pathogenic micro-organisms in a normally susceptible population can be limited. The overall increase in life expectancy in Europe over the past three centuries can be attributed primarily to higher standards of hygiene and nutrition, but the decreased frequency and magnitude of disease epidemics seen this century is largely due to the development and use of vaccines (Perkins, 1973). In western countries the incidence of many diseases has been reduced to insignificant levels and the world-wide eradication of one life-threatening disease, smallpox, has been achieved through a carefully co-ordinated intensive vaccination programme (*see* Behbehani, 1983).

A number of factors can influence the success of a particular vaccine. Of paramount importance is that the vaccine should be safe, effective in inducing high-level and long-lasting immunity and inexpensive enough to find broad application. Also of importance is the commitment and organization to deploy the vaccine in an efficient way. In developing countries the economics of large vaccination programmes has to be evaluated seriously when only a limited public health budget is available (Creese *et al.*, 1982). The mathematical literature dealing with the design of optimal vaccination programmes can be used to determine the efforts necessary to control and eradicate various diseases (Anderson and May, 1982).

Abbreviations: bp, base pairs; EF, edema factor; ETEC, enterotoxigenic *Escherichia coli*; FMDV, foot-and-mouth disease virus; HA, haemagglutinin; HBsAg, hepatitis B surface antigen; IG, immunoglobulin; LF, lethal factor; LPS, lipopolysaccharide; LT-toxin, heat-labile toxin; PA, protective antigen; ST-toxin, heat-stable toxin; VSV, vesicular stomatitis virus.

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Historically, vaccines have consisted of either (1) inactivated whole bacterial cells or viral particles; (2) sub-cellular components which have protective but not toxic activity; (3) inactivated sub-cellular components, e.g. toxoids, or (4) attenuated strains derived from pathogens or related micro-organisms which can establish a limited infection in the host sufficient to induce immunity but not full clinical disease symptoms. Until recently the options for vaccine design were limited by a number of factors, including rudimentary knowledge of the antigenic structure of micro-organisms and the lack of understanding of pathogenic mechanisms and the nature of the host's immune response. However, intensive research in several disciplines is leading to an increased understanding in these vital areas. The growth of molecular biology, the advent of recombinant DNA techniques and the use of monoclonal antibodies and immunological analysis is leading to a reappraisal of the approaches to vaccination and vaccine design. Increased knowledge of the antigenic structure of bacterial and viral pathogens has led in many cases to the identification and characterization of protective antigens which can be manipulated using biochemical or genetic techniques. As a direct consequence of these breakthroughs there is now an increasing demand for the production of vaccines with a more completely defined antigenic structure and composition, as opposed to a complex mixture of uncharacterized components, many of which may be non-essential and potentially harmful. Reducing the antigenic complexity of a vaccine is attractive because it reduces the likelihood of inducing side-effects in the host and opens up the possibility of creating multivalent vaccines which are effective against a range of pathogens.

In this review we will use specific examples to illustrate how molecular techniques are being used to investigate new approaches to vaccine design. From the work carried out to date it is clear that the problems of developing effective new vaccines are great but the potential of the molecular approach remains vast.

Immunological considerations

Vaccination is based on a deceptively simple observation: that exposure to a pathogenic entity can result in the host mounting a long-lasting immunological defence preventing reinfection by the same organism. Although a comprehensive review of immunology relevant to immunoprophylaxis is beyond the scope of this review it is a vital area of understanding for the successful application of genetic engineering to vaccine development. This is especially true as many of those working with the new technologies to develop vaccines do not have backgrounds in the immunological sciences. For more detailed introductions to immunology there are many excellent reviews and texts including those by Roitt (1977), Fundenberg *et al.* (1978), Playfair (1979), Ada (1982) and Bomford (1983).

LYMPHOCYTES AND IMMUNITY

Adaptive immunity, a major component of the overall antimicrobial immunity at which vaccination is aimed, is based on the specialized properties of

lymphocytes which can respond to foreign antigens, which may be proteins, carbohydrates or lipids, by the production of specific antibodies. Lymphocytes are small cells found in the blood which search out foreign material by recirculating through tissues and back to the blood via the lymphatic system. There are two main types of lymphocyte: both are formed in the bone marrow, but one type (B) enters the lymph nodes directly, whereas the other type (T) reaches these nodes only after a period of residence in the thymus. B lymphocytes recognize individual antigens through their specialized surface receptors of immunoglobulin, which T lymphocytes do not possess. The B lymphocytes have a long life span and are stimulated by the presence of an antigen to divide into numerous plasma cells of identical specificity, which synthesize one particular antibody. In conjunction with the recirculation of plasma cells and lymphocytes, this ensures that specific 'memory' following a local response has a bodywide distribution.

Generally antigens stimulate B cells to divide into antibody-producing plasma cells only if T cells are also present. Although most antibody responses involve collaboration between B cells and 'helper' T cells, the nature of the interaction between them and the phagocytic cells (macrophages) which first take up antigens, is still not clear. Some T lymphocytes actually suppress immune responses and regulation of such responses appears to depend on a balance between 'help' and 'suppression' by T lymphocytes (*see also* 'Cell-mediated immunity').

Presenting the antigen to the immune system in an optimal manner is an important feature of new vaccine design. Antigens may require help from macrophages in order to trigger lymphocytes to proliferate. The nature of this help is not clear but may involve antigen binding to surface receptors or some processing by macrophage enzymes. There is also the possibility that antibody, once formed, can further assist macrophages in antigen presentation. A second exposure to the same foreign antigen results in a quicker and stronger secondary immune response.

HUMORAL IMMUNITY

Lymphocytes are genetically programmed to respond to a single antigen. However, the antibodies that are produced following specific stimulation by exposure to antigen may be any of a range of immunoglobulin (Ig) types. The different classes of immunoglobulins (IgA, IgM, IgD, IgE, IgG) have peculiar physical and biological properties which determine their function in immunity.

The most abundant and widespread class of immunoglobulins, IgG, is found in both intravascular and extravascular compartments, in most secretions and in the milk and intestine of ruminants. On re-exposure to antigen the IgG antibodies are the major component of the secondary response. The transfer of IgG antibodies from mother to offspring is the basis of producing passive resistance in neonates. This is important in disease resistance as the immune system of newborns is not immediately capable of a full response to antigenic challenge.

For most protein antigens, IgM antibodies are among the first to appear and are often transient, being replaced by IgG antibodies after only a few weeks.

Production of IgM by plasma cells derived from B lymphocytes is least dependent on 'help' from T lymphocytes. Both IgM and IgA production may occur locally rather than systemically in response to antigens that enter through the mucosa. Such secretory antibodies may have a role in preventing the first stages of infections acquired through mucous membranes by preventing attachment or adherence of micro-organisms and blocking their establishment and further proliferation. Critical to the design of new vaccines is the observation that production of secretory IgA requires a local antigenic stimulation. The same antigen administered parenterally would result only in the production of circulating IgG antibodies, with little effect on mucosal and intestinal infections where secretory IgA may be the first line of immunological defence.

CELL-MEDIATED IMMUNITY

Cell-mediated immunity is also an adaptive response but does not involve circulating antibodies. The lymphocytes which are involved in cell-mediated immunity include several types of specialized T cells which are distinct from the helper T cells mentioned previously. After exposure to a specific antigen, T cells become activated through clonal selection and proliferation. Upon re-encountering the homologous antigen the primed T cells can release lymphokines — soluble factors with a variety of defence functions such as macrophage-activating factor and mitogenic factors which can increase the destructive efficiency of macrophages and other non-lymphoid cells. Cytotoxic T cells are also capable of directly lysing virus-infected cells. There is often an inverse relationship between the cell-mediated and humoral antibody responses which may be due to regulatory interactions, through suppressor T cells that inhibit the functions of other lymphocytes, as well as to direct blocking of the cell-mediated response by antigen–antibody complexes.

ANTIMICROBIAL IMMUNITY

In some instances, antibodies can function directly by combination with the antigen, as in toxin neutralization, but generally act in combination with complement and phagocytic cells to remove microbes in one of two ways: (1) invading micro-organisms can directly activate complement by the 'alternative pathway' leading to their lysis or phagocytosis; (2) the same effect may also occur when the complement component C3 is activated by specific antibodies. The complement component C3 together with antibody may induce attachment of the microbe to the phagocytic cell through its C3 or Fc receptors (opsonization) and so enhance phagocytosis by leucocytes or macrophages.

Whereas complement and antibody are most active against those micro-organisms free in the blood or tissues, the cell-mediated responses are generally more active against those which reside within host cells. However, the form of immunity which is most successful against the pathogen will depend on the strategies followed during the course of infection. Most parasites have adopted tactics which enable them to evade, resist or inhibit immune

mechanisms. Many animal vaccines are thought to act through cell-mediated immunity because protection is not correlated with antibody levels in blood and body fluids.

ADJUVANTS

The immune response can be enhanced by the addition of materials called adjuvants together with the antigen (for reviews *see* Edelman, 1980; Bomford, 1984). Adjuvants are a diverse collection of materials with a variety of poorly understood mechanisms for stimulating T cells, B cells or macrophages, or for assisting in a correct presentation of the antigen to the immune system. Among those compounds which act by binding or retaining antigen to prolong its antigenicity are oil emulsions, insoluble inorganic compounds such as aluminium hydroxide and synthetic lipid vesicles or liposomes. One of the most powerful adjuvants, Freund's Complete Adjuvant, consists of a mineral oil plus emulsifier, to ensure a more uniform mixture with antigen combined with dead *Mycobacterium*. The use of this adjuvant can boost antibody titres by several orders of magnitude, especially with subunit vaccines.

Unfortunately, the use of Freund's adjuvant causes severe local inflammation and is unsuitable for use in man. Other adjuvants must then be used. Adsorption to aluminium hydroxide is commonly used in human vaccines and is applicable to subunit vaccines, especially with synthetic peptides where a carrier can be chosen which is well adjuvantized by this compound. Some recent progress with new adjuvants such as muramyl peptides (Chedid *et al.*, 1982), when combined with advances in the use of synthetic peptides, holds out promise for totally synthetic vaccines without separate adjuvant.

Not only do adjuvants enhance the immune response but they can also modify it in qualitative ways. Immunodominant sites on an antigen may be expressed differentially, or the balance between humoral and cell-mediated immunity may be altered.

IMMUNOGENICITY

What is required for a molecule to stimulate an immune response following injection? First of all it must contain foreign antigenic determinants for the immune system to recognize. Secondly, the foreign antigen must be relatively large. Small peptides and monosaccharides are not immunogenic on their own but may be when part of a larger molecule. This size requirement may be a simple consequence of the necessity for simultaneous recognition and binding of the antigen by both T and B lymphocytes.

For some antigens, such as polysaccharides, which consist of a series of repeated identical antigenic determinants, the binding of antigen alone may be sufficient to trigger B lymphocyte differentiation. However, for most antigens the participation of a second type of lymphocyte, the helper T cell, is required. Helper T lymphocytes have cell-surface receptors which interact with the antigen and stimulate B cells to start antibody synthesis. Macrophages are also involved in the initiation of the immune response through antigen binding and

presentation on the macrophage surface, possibly in association with histocompatibility antigen.

The requirements for immunogenicity have usually been met in conventional vaccines containing whole live attenuated or inactivated micro-organisms as well as in toxoid vaccines, although the latter may require adjuvants. Where the aim of genetic engineering technology is to produce pure viral or bacterial antigens, then significant problems arise in enhancing the immunogenicity of the product. Purified cellular components are generally not as immunogenic in isolated form as they would be when assembled in the complete micro-organism. This is clearly the case for foot-and-mouth disease virus (FMDV) and polio virus where isolated structural proteins are poor immunogens.

Where the complete structural protein fails to induce an antibody response it may still be possible for small fragments or peptides to be effective. It may then be necessary to conjugate the peptides to a carrier protein to provide an antigen of high molecular weight and to provide determinants recognizable by helper T cells. It may be possible biosynthetically to produce hybrid proteins with these properties.

Live virus vaccines stimulate good antibody responses without the need for powerful adjuvants. The engineering of live hybrid viruses carrying foreign antigenic determinants will clearly have an important role in new vaccine development.

The contribution of genetic engineering

In part, the recent growth in interest in developing new vaccines arises from the many possibilities generated by the techniques of genetic engineering. The techniques of molecular cloning have brought about a scientific revolution that has spread to laboratories around the world. As many of those who are working in this field are not conversant with genetic engineering techniques, it is necessary to review the present state of the field to point out some of the many possibilities that have been created, as well as the limitations that currently exist. There are many ways in which recombinant DNA techniques are of value:

1. To analyse genomes of pathogens as a step in characterizing important aspects of antigen structure and heterogeneity, especially for surface proteins that may be targets of immunity;
2. To produce, in *E. coli*, proteins of pathogens which are otherwise available only in limited quantities — especially where the pathogen is difficult or impossible (or dangerous) to culture *in vitro*. Such proteins can be valuable research tools and can be developed into diagnostic reagents or vaccines.

In this regard, genetic engineering can offer means of vaccine production, but even when traditional vaccine technologies are maintained, genetic engineering can improve understanding of the organism and its immunologically active components, as well as offering means of quality control. Genetic engineering may also assist in introducing a new generation of live vaccines by determining the mechanisms of attenuation in vaccine strains and so indicating

how new, improved strains might be constructed. There is now the possibility of producing heterologous live vaccines, in which a non-pathogenic self-replicating virus or bacterium may carry one or more foreign antigenic determinants which can be administered directly and provide long-lasting immunity against one or more pathogenic micro-organisms.

MOLECULAR CLONING

The essential feature of genetic engineering (or recombinant DNA) technology is that it enables one specifically to transfer genetic information from one organism into another. Molecular cloning techniques are based on introducing DNA from micro-organisms or higher eukaryotes into *E. coli*. It is now possible to transfer DNA into a range of bacteria, yeast or mammalian cells but *E. coli* remains the organism of choice for most studies. A wide variety of plasmid and bacteriophage vectors have been developed which can be used in *E. coli*. For cloning very large fragments of DNA cosmid vectors are well suited. A cosmid combines useful features of both phage and plasmid vectors. A plasmid containing the *cos* site of bacteriophage lambda (cosmid) can be packaged, with up to 40 kilobases of foreign DNA, into infective particles by lambda coat proteins. Once inside the bacterium, the cosmid replicates as a plasmid. A collection of 200 cosmids can represent an entire bacterial genome. Mammalian genomes are considerably larger and a cosmid bank of 10^5–10^6 separate clones would be required. Bacteriophages have also been very successfully used for constructing gene banks. Because of the very large numbers of clones that need to be examined for large genomes it may be preferable to make gene banks from expressed genes. Thus for eukaryotic cells and certain viruses a process called cDNA cloning is used. This involves the enzymatic conversion of polyadenylated messenger RNA into double-stranded DNA and subsequent insertion into a bacterial plasmid (for reviews *see* Efstratiadis and Villa-Komaroff, 1979; Williams, 1981).

IDENTIFICATION OF RECOMBINANT CLONES

Having generated genomic or cDNA gene banks it is necessary to identify the individual clone coding for the protein of choice. This can be the most difficult step for some genes. There are three general ways of identifying the desired recombinant clones.

Where there is pre-existing knowledge of the nucleic acid sequence, or purified mRNA is available, it is possible to detect *in situ* hybridization between bacterial colonies or bacteriophage plaques and specific probes of ^{32}P-labelled DNA or RNA. This is a simple and fast technique. This technique can be used even where there is no knowledge of the nucleic acid involved but where at least a partial amino-acid sequence of the protein is known. Knowing the genetic code, a range of potential corresponding nucleic acid coding sequences can be determined. Unfortunately the degeneracy of the genetic code, in which a single amino acid may be specified by several different triplet codons, means

that a large number of sequences are possible and a complex oligonucleotide mixture may need to be synthesized to enable one to identify the desired clone.

A second way of identifying clones is through hybrid selection. Cloned DNA molecules are denatured, immobilized on a solid matrix and hybridized to preparations of mRNA. Only the mRNA that corresponds to the clone will be retained. This mRNA can be separated from the DNA and translated. The protein that is formed by transcription and translation from this mRNA can be further identified by immunoprecipitation.

A third approach to identifying recombinant clones is through direct immunological screening of the gene bank. This approach has been developed and refined over the past few years and is invaluable where the only means of characterizing the desired protein is through antisera or monoclonal antibodies (Sanzey *et al.*, 1976; Skalka and Shapiro, 1976; Broome and Gilbert, 1978). Specific vectors have been designed to enhance the ease and sensitivity of immunological screening.

SEQUENCING AND GENETIC ANALYSIS

Having obtained a clone of the desired gene, the next step is to determine its structure and properties on a molecular level. Here the breakthrough came with the development of techniques which allow one to determine the exact nucleotide sequence of the cloned insert (Maxam and Gilbert, 1977; Sanger, Nicklen and Coulson, 1977). Knowledge of the nucleotide sequences allows one to predict the amino-acid sequence of the encoded protein. This is particularly important where the protein is not available in sufficient quantities to allow the amino-acid sequence to be determined by chemical techniques. Additionally one may obtain information on the regulation and processing of the protein in question. For example the molecular basis of antigenic variation in trypanosomes has been partly elucidated by such techniques.

Expression of foreign genes in *E. coli*

Where the goal of genetic engineering is to produce proteins in *E. coli* then it is usually necessary to carry out a number of alterations to the originally cloned gene to enable high levels of expression to occur. The gene will need to be precisely modified and inserted into expression vectors (Roberts and Lauer, 1979; Guarante, Roberts and Ptashne, 1980) because the foreign gene does not carry the appropriate promoter and other control signals needed for expression in bacteria. Expression vectors contain promoter sequences of DNA that are required for the efficient transcription of their mRNAs in *E. coli*. Such vectors are essential for expressing eukaryotic genes in *E. coli* but are also useful for increasing production of prokaryotic gene products. Although almost all expression vectors are plasmid based there are now examples of the use of bacteriophage expression vectors as well (Young and Davis, 1983, Siggens *et al.*, 1983).

The basic requirements for expressing a gene in *E. coli* are as follows:

1. The coding region of the gene must not be interrupted by intervening sequences. Intervening sequences occur in many eukaryotic genes and are spliced out by specific processes which do not occur in bacteria.
2. The gene must be placed under the control of an *E. coli* promoter that is efficiently recognized by the *E. coli* RNA polymerase. Eukaryotic promoters have little or no activity in bacteria so the homologous bacterial regulatory signals must be used.
3. The mRNA that is synthesized must also have further sequences to allow ribosomes to bind at the correct place and initiate translation.
4. The protein must be stable and not subject to the cytoplasmic proteases present in *E. coli*. There are further requirements which may need to be met to ensure the highest possible levels of expression. Some of these factors will be discussed in greater detail.

PROMOTERS AND THE INITIATION OF RNA SYNTHESIS

Promoters are DNA sequences that direct RNA polymerase to bind to DNA and to initiate RNA synthesis. Strong promoters result in a high frequency of initiation of RNA synthesis. Polymerase interaction with the DNA is a complex multistep process so the initiation step is probably rate limiting in the overall rate of RNA synthesis. Eukaryotic promoters are not active in *E. coli*. There is even significant variation in promoter features between different bacteria.

Examination of a number of *E. coli* promoters has revealed two highly conserved areas. One of these regions is located about 10 base pairs (bp) from the point at which transcription starts and is called the −10 region or Pribnow box (Pribnow, 1975). The second area is positioned about 35 bp from the transcription start site and is referred to as the −35 region (Rosenberg and Court, 1979). Both regions are important in determining promoter strength because mutations that decrease the frequency of transcription usually decrease the extent of homology of the promoter sequence to the consensus sequence. Nevertheless, sequences outside the two conserved regions may also contribute to promoter strength.

There are several naturally occurring *E. coli* promoters that have been used to express foreign proteins such as *lac*, *trp* and the leftward promoter of bacteriophage lambda. There are now also examples of novel *in vitro* constructed hybrid promoters, such as the *tac* promoter, which are useful for expression (de Boer *et al.*, 1982). The most useful promoters are those that not only are strong but are regulated. The previously mentioned promoters can all be repressed or induced depending on the host strain and culture conditions. A fully induced promoter may lead to plasmid instability through interference with the replication mechanism. In addition, the accumulation of large quantities of foreign proteins may be harmful to the bacterium, leading to selection against the presence of the plasmid. Such problems can be quite serious in large-scale fermentations so it is desirable to be able to regulate the time of synthesis of the foreign protein.

INITIATION OF PROTEIN SYNTHESIS

Having produced high levels of mRNA in *E. coli*, the next step is the efficient translation of the specific mRNA by ribosomes to produce the desired protein. Thus the mRNA must contain a nucleotide sequence that functions as a ribosome-binding site. The typical *E. coli* ribosome-binding site includes a specific sequence of three to nine nucleotides in length spaced three to eleven nucleotides upstream of the initiation codon (AUG). This sequence is referred to as the Shine–Dalgarno sequence and is complementary to the 3′ end of the *E. coli* 16S rRNA. Base-pair interactions between the rRNA and the Shine–Dalgarno sequence are presumed to promote ribosome binding to the mRNA, leading to the initiation of translation.

Details remain to be worked out for the degree of complementarity, spacing and sequence surrounding the Shine–Dalgarno region that would give maximum rate of protein synthesis. A major complication is that ribosomes not only interact with this region upstream of the coding sequence, but are large enough to overlap (and interact with) up to 20 nucleotides downstream of the start codon. An examination of 74 ribosome-binding sites in *E. coli* suggested that, while there is no absolute requirement for a specific base at any given position, there is a marked preference for certain bases along the whole length of the RNA within the ribosome-binding domain, even though nearly half of that length includes translated codons (Scherer *et al.*, 1980). This places restrictions on the choice of codons for the first five or six amino acids and may even limit the choice of amino acids that can lead to high levels of translation. A further complication is introduced through possible secondary structures in the mRNA which may either enhance or inhibit ribosomal interactions.

LIMITS TO FOREIGN GENE EXPRESSION IN *E. COLI*

Not all of the variables have been worked out that would lead to maximum levels of expression of foreign genes in *E. coli* (e.g. 20% of total cell protein). The genetic engineer cannot guarantee that any protein can be produced at the desired level. Possibly the most serious problem is that foreign proteins in *E. coli* are in an 'unnatural' environment and may not fold or associate correctly. Such proteins may be degraded by the proteases present in *E. coli* and may not accumulate within the cell, even though their rate of synthesis remains high. One solution to this problem is to produce a hybrid or fusion protein. This is accomplished by making a fusion between the foreign gene and a gene for a stable bacterial protein. This may allow stable accumulation, often as an insoluble aggregate, but the fusion protein may not have the same biological activity as the native protein. Furthermore *E. coli* cannot glycosylate or modify proteins, as may naturally occur in eukaryotic cells. Again, this may lead to inactive or degraded proteins. For such reasons *E. coli* may be an unsuitable host for the production of some foreign proteins.

ALTERNATIVE HOSTS FOR GENETIC ENGINEERING

Bacteria

There are now several alternative hosts for genetic engineering experiments where *E. coli* may be unsuitable. Other bacteria such as *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Methylophilus methylotrophus* have been used successfully for cloning and/or expression studies. In particular it was hoped that bacteria could be persuaded to secrete foreign proteins into the extracellular media for greater ease of purification but this has proved to be difficult (Henning *et al.*, 1983; *see also* Chapter 5 of this volume).

Yeast

Yeast cells have also been exploited for gene-expression studies and theoretically combine the technical advantages of working with unicellular organisms with the eukaryotic features that are lacking in *E. coli* (particularly glycosylation). Indeed, the expression of hepatitis B surface antigen which has been difficult to achieve in *E. coli* has now been accomplished in yeast by several groups (Valenzuela *et al.*, 1982). Yeasts are very likely to have an important role in the production of recombinant proteins suitable for vaccines.

Mammalian cells

Even yeast cells cannot carry out all of the processing, glycosylation and assembly reactions that may occur in mammalian cells. There are some proteins that may need to be synthesized in mammalian cells in order to have all the correct properties and conformation to be a successful immunogen. It is not yet clear that hepatitis surface antigen synthesized in yeast has all the same properties as that made in mammalian cells. If they are essentially indistinguishable, then the yeast-derived material would be the product of choice as the fermentation would be simpler and less expensive. Mammalian-cell technology may not be suitable where very large quantities of protein antigen are required.

Live vaccines

The engineering of bacteria or viruses to produce live vaccines is an important extension of recombinant DNA technology and will be discussed in subsequent sections of this review (*see also* Chapter 14 of this volume).

Subunit and synthetic peptide vaccines

The present generation of whole-cell vaccines has diminished the incidence of and mortality from numerous infectious diseases. However, for many other diseases vaccines are not available or are unsatisfactory because of the poor levels of protection generated or the presence of side-effects following vaccination. Killed or live attenuated microbial agents generally contain a large number of components (antigens) which are presented to the immune system.

Such antigens tend to be part of the morphological structure of the micro-organism and may include proteins, glycoproteins, carbohydrates and complexes of lipids with proteins and polysaccharides.

SUBUNIT VACCINES

Although a number of antigens are recognized by the immune system, not all of the antibodies that bind to the micro-organism and its extracellular products will necessarily lead to its destruction by host systems. The viral or bacterial neutralizing antibodies that are generated by whole-cell vaccines may be attributable primarily to only one or a few antigens. The basis of developing subunit vaccines is to identify the essential immunogenic components of a virus or bacterium and to devise a way of producing the particular antigen separately from the rest of the cellular components which do not contribute to the protective immune response. The immune system could then be presented with a simple immunogen which should produce high levels of specific neutralizing antibody but without the side-effects or hazards associated with some whole-cell vaccines. Although this may seem to be a daunting task, the past decade has already seen the development of effective subunit vaccines against some meningococci and pneumococci by isolating the polysaccharides from the bacteria (Austrian *et al.*, 1976). Progress has been made for other bacteria such as *Haemophilus influenzae* and streptococci but further work is needed to enhance the immunogenicity of most subunit vaccine preparations.

Subunit vaccines produced by isolating surface components of viruses are well advanced for hepatitis and herpes. However, for many viruses for which subunit vaccines are desirable, it has not been possible to produce enough virus to provide adequate levels of starting material to isolate individual components. Genetic engineering techniques then become essential, as proteins produced in *E. coli*, yeast or mammalian cells could be available in virtually unlimited amounts.

The four steps necessary for the development of a genetically engineered subunit vaccine are:

1. Identification of the protective immunogen(s) on the micro-organism;
2. Molecular cloning of the gene for the candidate antigen;
3. Expression of the antigen in *E. coli* or other host systems;
4. Determination of the immunological activity of the final product and possible enhancement of its immunogenicity.

The development and use of monoclonal antibodies has been a major contribution to the identification and purification of specific components out of the complex mix of antigens present on the surface of micro-organisms. The use of highly purified components is necessary for understanding the antigenic structures important in immunization.

SYNTHETIC PEPTIDE VACCINES

A further refinement to the subunit vaccine concept follows from the observation that only small portions of a native antigen are exposed to the

immune system. Studies of many globular proteins have concluded that there is on average only one antigenic determinant per 5000 daltons of protein (equivalent to about 40 amino acids). Of the limited number of antigenic sites present, only a few immunodominant ones may be involved in the neutralizing immunity. Provided that an individual determinant can be isolated and retain its immunogenic properties, it should be possible to produce a highly specific vaccine free from competing or non-essential antigenic components. The basis for using synthetic antigens for vaccination was laid down in studies by Ruth Arnon and Michael Sela (Arnon *et al.*, 1971; Langbeheim, Arnon and Sela, 1976) and has since been confirmed and expanded by many other research groups (*see* Lerner, 1982; Sutcliffe *et al.*, 1983).

The first step in developing a synthetic peptide vaccine is to identify the relevant immunogen and to determine its amino-acid sequence. This can be accomplished either through direct chemical sequencing or by knowledge of the nucleic-acid sequence of its structural gene. The advancement of molecular cloning and DNA-sequencing technology is such that structural information about a protein is now most easily obtained from analysis of its genetic structure. Knowing the linear sequence of amino acids, the next step is to identify the principal antigenic and immunogenic sites. There are a variety of ways in which this can be approached and several techniques should be applied to the problem in order to generate a model that can be viewed with confidence.

The chemical and enzymatic cleavage of purified proteins and subsequent analysis of their immunological properties was successfully used to elucidate the complete antigenic structures of myoglobin and lysozyme (Atassi, 1977; Atassi and Habeeb, 1977). Unfortunately this approach required milligram quantities of purified proteins and an average of ten years' research for each protein. Clearly this is not practical for many proteins which are available only in small quantities. Crystallographic studies of the three-dimensional structure of proteins are also of assistance in determining residues which are on the surface of the macromolecule but, again, such studies are lengthy and can require large amounts of highly purified protein. Furthermore, the identification of exposed residues is not sufficient to define the immunogenic determinants.

A powerful approach is to use monoclonal antibodies with neutralizing activity to identify the smallest components of the antigen which are still capable of specific binding activity. Fragments of the antigen suitable for such testing could be produced by chemical or enzymatic breakdown of the antigen, by chemically synthesizing peptides or by expressing only portions of the antigen in *E. coli* (van der Werf *et al.*, 1983). Specific plasmid vectors for the antigenic analysis of proteins have been developed (Rüther *et al.*, 1982). As more information on the antigenic structure of proteins becomes available there have been considerable efforts made to develop methods of predicting protein conformation, and thus important antigenic determinants, solely from knowledge of the amino-acid sequence.

Charged or hydrophilic residues are more likely to be on the surface of a protein. By analysing the amino-acid sequence to find points of highest local average hydrophilicity one finds regions which are located in, or immediately adjacent to, an antigenic determinant (Hopp and Woods, 1981). However,

some antigenic determinants are not correlated with hydrophilicity: such predictions therefore can assist in choosing regions to examine but cannot be solely relied upon. Antigenic sites that are hydrophobic have been reported for the haemoglobin alpha-chain (Kazim and Atassi, 1982) and for influenza haemagglutinin (Atassi and Webster, 1983).

These techniques will enable one to identify antigenic sites. This type of analysis does not guarantee that these sites, in isolation, will have immunogenic activity. The critical test is examination of the antibody response in animals following experimental vaccination with the compound. Failure to generate neutralizing antibodies does not mean that the region selected is not an immunogenic site. The peptide may require special carriers or adjuvants to encourage it to fold into the proper conformation and thus mimic its shape when it is part of a larger structure on the virus or bacterial surface. The present level of knowledge of peptide conformation does not allow one to do this in a systematic way. Recent experiments have suggested that short peptides have constrained conformations, especially after coupling to a carrier, and that the conformation adopted by the free peptide has a high probability of resembling that taken by the same region when part of an intact protein (Niman *et al.*, 1983). Although this is an encouraging observation, it does not mean that synthetic peptides will generate antibodies of sufficient affinities and in sufficient quantity to be used as vaccine immunogens. These points are further amplified by consideration of specific examples of new vaccine developments.

Viral subunit vaccines

FOOT-AND-MOUTH DISEASE

Foot-and-mouth disease virus (FMDV) is an excellent example of progress in this field as the use of recombinant DNA technology for new vaccine development has progressed further with FMDV than for almost any other virus. For a more detailed analysis see the review by Cheung and Küpper (1984) in volume 1 of this Series.

The picornavirus FMDV is the cause of an economically devastating disease of cloven-hoofed animals. The disease is currently controlled through slaughter in countries where outbreaks are infrequent and by vaccination in countries where it is endemic. Vaccination has successfully reduced outbreaks of the disease and complete eradication of the virus from some countries has been achieved. Currently worldwide annual production of FMD vaccine is running at 1000 million doses produced by inactivating virus grown in baby hamster kidney cells or bovine tongue epithelia.

One major problem in the production of effective vaccines against FMDV is the existence of many antigenic variants of FMDV, with seven main serotypes and over 60 immunologically distinguishable subtypes. Despite this problem, carefully selected trivalent vaccines are able to give sufficient protection in most countries. There are several reasons for attempting to develop a novel FMD vaccine. Apart from the general hope that recombinant-DNA technolo-

gy could make a 'cheaper and more effective' vaccine, there are several specific problems associated with the present inactivated virus preparations:

1. The virus strains used for vaccine production are fully virulent and require meticulous techniques to ensure their complete inactivation;
2. Virus particles are unstable and require refrigeration during transport and storage to maintain immunogenicity;
3. Some virus subtypes grow poorly in BHK suspension cultures and cannot be used in vaccine preparations because of the small yields of antigen produced. In addition, strains adapted to grow well in culture may differ antigenically from the field strain.

FMDV has a single-stranded positive-sense RNA genome of about 8000 nucleotides coding for a single primary translation product which is processed by proteolysis into several precursors. One of these is the capsid precursor p88 which is processed by viral encoded protease(s) into the four structural polypeptides VP1, VP2, VP3 and VP4. Early experiments suggested that VP1 was the only capsid protein able to induce neutralizing antibodies in guinea-pigs and was likely to carry the major virus antigenic determinants (Laporte *et al.*, 1973; Bachrach *et al.*, 1975).

The prospect of producing a subunit vaccine based on the capsid protein VP1 was a stimulus to apply the techniques of recombinant-DNA technology to this problem. By 1981 three groups had reported the successful cDNA cloning of FMDV capsid protein (Boothroyd *et al.*, 1981; Kleid *et al.*, 1981; Küpper *et al.*, 1981). It is possible to produce, in *E. coli*, large quantities of VP1 fused to bacterial proteins. Using strong bacterial promoters up to 10^6 molecules of fusion protein per bacterial cell can be made (Kleid *et al.*, 1981). However, the VP1 fusion proteins are insoluble and aggregate within the cell. Although this probably protects the protein from proteolysis, such insoluble proteins may not be the most suitable for vaccine development.

There are now several publications showing that bacterially produced VP1 fusion proteins can induce virus-neutralizing antibodies in guinea-pigs, cattle and swine. Cattle and swine were protected after two injections of 250 μg VP1 fusion protein (serotype A, subtype 12, strain 119) using an oil adjuvant (Kleid *et al.*, 1981). For field use it is essential that the vaccine is effective after a single injection. It is therefore encouraging that the same fusion protein has been shown to produce significant levels of neutralizing antibodies in guinea-pigs after only a single injection of 100 μg (Moore, 1983). However, in the same experiment carried out with a different serotype (O_1 Campos), VP1 fusion protein was less successful and only low levels of neutralizing antibodies were produced.

Molecular cloning has generated a greater understanding of the nature of antigenic variation in FMDV. From cDNA-cloning experiments complete sequences are now available for VP1 from several different serotypes and subtypes. There is a 60–72% homology between the nucleic-acid sequences for VP1 of different serotypes and 88% or higher homology within a single serotype (Makoff *et al.*, 1982; Beck, Feil and Strohmaier, 1983). The variation is concentrated in a number of discrete regions corresponding to amino acids

42–60, 129–160 and 193–204. Cleavage of virus and virus-derived VP1 generates a number of overlapping fragments which have been used as immunogens (Strohmaier, Franze and Adam, 1982). This approach identifies two regions, amino acids 138–154 and 200–213 (carboxy terminus), which overlap with two of the variable regions and so may carry the main immunogenic determinants of VP1. This conclusion is confirmed by reports that synthetic peptides corresponding to these regions, when coupled to a suitable carrier and injected with adjuvant, can give rise to virus-neutralizing antibodies (Bittle *et al.*, 1982, Pfaff *et al.*, 1982). The further observation that synthetic peptides mimic the immunological subtype specificity of whole virus shows that one of the principal neutralizing determinants has been identified by this range of techniques (Clarke *et al.*, 1983).

Although bacterially-produced VP1 can give rise to virus-neutralizing antibodies and can even protect animals against virus challenge, such preparations cannot compete with the present vaccines in cost and efficacy. Synthetic peptide vaccines may be more promising for FMDV but clearly need further development to reduce their costs, to increase their ability to stimulate the immune system and to increase the cross-protection produced for related virus strains. For commercial acceptance any new vaccine will need to have clear advantages over the present inactivated virus vaccine. The fact that a new vaccine is not yet available shows that, despite the considerable work towards this goal, the production of an effective subunit vaccine of high immunogenicity is more difficult than expected.

POLIO

Poliovirus, the causative agent of poliomyelitis, is a picornavirus with many similarities to the foot-and-mouth disease virus discussed previously. The virus is composed of a positive-strand RNA genome enclosed in a capsid containing 60 copies of each of the four structural polypeptides VP1, VP2, VP3 and VP4. Despite many years of successful vaccination with both live attenuated and inactivated virus vaccines, the basis for the induction of virus-neutralizing antibodies is only now beginning to be understood.

One of the difficulties in identifying the antigenic sites of poliovirus is that neutralizing antibodies raised against native virus (D particles) do not react with any of the isolated capsid polypeptides. Similarly, the coat proteins generate only very low levels of neutralizing antibodies after inoculation into animals. The possibility that the principal antigenic sites were conformational or discontinuous frustrated attempts to carry out standard immunochemical studies on the virus.

The eventual identification of VP1 as the polypeptide carrying neutralizing epitopes (antigenic determinants) was accomplished by cross-linking neutralizing antibodies to the intact virus (Emini *et al.*, 1982). Two epitopes were localized on VP1 by this technique. The detailed identification of one of these sites has been carried out by groups using two very different techniques. Van der Werf *et al.* (1983) expressed poliovirus type 1 VP1 in *E. coli* fused to a bacterial protein. This hybrid protein could be immunoprecipitated by a

virus-neutralizing monoclonal antibody raised against heat-inactivated virions (whole virus particles). By generating a series of deletions within the coding sequence of the fusion protein it was possible to locate the neutralization epitope in the domain between amino acids 95 and 110 of VP1.

Poliovirus peptides for type 1 virus corresponding to residues 11–17, 70–75, 70–80, 93–103 and 97–103 were tested for their ability to act as immunogens (Emini, Jameson and Wimmer, 1983). Only one peptide (93–103) had any neutralizing activity but all five did prime the immune system for a long-lasting virus-neutralizing IgG antibody response following a single inoculation of low levels of poliovirus. It is not known whether peptide-mediated priming of the immune system can protect a mammal against naturally acquired infection but this may be possible with diseases such as polio or hepatitis which have a long incubation period.

A more precise localization of this important antigenic site was achieved by examining antigenic mutants of poliovirus type 3 which were selected for resistance to neutralizing monoclonal antibodies. Several of these viruses had point mutations in the genome which altered the VP1 polypeptide in the region of amino acids 93–98 (Minor *et al.*, 1983). A further examination of additional antigenic mutants (Evans *et al.*, 1983) demonstrated that virtually all of the mutations conferring resistance to neutralization are confined to an eight-amino-acid sequence of VP1 (amino acids 93–100). The excellent correlation of this finding with the region identified by van der Werf *et al.* (1983) is a great stimulus to the design of synthetic peptide vaccines. The experimental identification of this antigenic site is of considerable importance as it would not have been easily predicted on the basis of hydrophilicity or theoretical structural analysis alone. The region from 93 to 100 is not the most variable region when comparing type 1 and type 3 poliovirus sequences, but the sequence variation that is found in this area could well account for serotype specificity. Further studies of peptides from poliovirus are likely to be hampered by the low immunogenicity of such compounds when compared with the inactivated virus immunogens.

HEPATITIS

The impetus to develop a genetically engineered vaccine for hepatitis is strong and has attracted the attention of many research teams. In contrast to both FMDV and polio where excellent vaccines already are in use, the existing hepatitis B vaccine is expensive and available in relatively limited quantities. Because the virus cannot easily be cultured it has been necessary to purify subviral components from the plasma of chronically infected donors. Genetic engineering techniques offer the promise of cheap vaccine immunogens in virtually unlimited quantities.

The gene for the principal component of the virus surface, hepatitis B surface antigen (HBsAg), has been cloned and antigenic material expressed in bacterial, yeast and mammalian cells. As expression of HBsAg in *E. coli* has proved to be difficult (Burrell *et al.*, 1979) it has been necessary to use yeast (Valenzuela *et al.*, 1982) or mammalian cells (Dubois *et al.*, 1980; Moriarty *et*

al., 1981) for high levels of production of HBsAg protein. Surface antigen produced in either expression system can assemble into 22 nm particles similar to those found in infected human plasma. Both sources of surface antigen produce a highly immunogenic polypeptide even though glycosylation occurs only within the mammalian cells and not in yeast. This is an encouraging observation as the necessity to attach the correct carbohydrate residues to glycoprotein antigens would severely limit the application of recombinant DNA techniques to a number of antigens.

Purified yeast-derived surface-antigen particles injected together with aluminium hydroxide adjuvant stimulate the production of virus-neutralizing antibodies in monkeys and chimpanzees (McAleer *et al.*, 1984). More importantly, the vaccinated chimpanzees were totally protected against challenge by both homologous and heterologous virus subtypes. The yeast-produced HBsAg is likely to be more economic than the material derived from mammalian cells and human clinical trials are already in progress with such material.

As an alternative strategy to vaccine development, considerable effort has been expended on synthesizing peptides that carry the antigenic determinants of HBsAg. Because of the considerable uncertainties in predicting antigenic sites a series of peptides covering most of the 220 residues were synthesized and tested in rabbits (Lerner *et al.*, 1981). Antisera against several of the peptides were capable of reacting with intact surface antigen, with the most effective peptide corresponding to amino acids 95–109.

An entirely different region was identified following computer analysis of secondary structure and hydrophobicity (Dressman *et al.*, 1982). The region chosen for study, position 117–137, contains two cysteine residues which could form a disulphide loop. The cyclized peptide was immunogenic even in the absence of a carrier protein.

A third region of the HBsAg was investigated by three separate groups. A peptide corresponding to amino acids 138–149, selected by theoretical analysis of surface antigen structure, could induce the formation of anti-HBsAg antibodies (Hopp and Woods, 1981). The same region was identified as an important site by Bhatnagar *et al.* (1982) and by Neurath, Kent and Strick (1982). A detailed analysis of the immune response to a peptide encompassing residues 135–155 showed that only a subpopulation of the antipeptide antibodies was capable of reacting with HBsAg (Neurath, Kent and Strick, 1982). The equilibrium constant for the reaction of antipeptide antibodies with HBsAg was one hundredfold lower than that for peptide recognition and was well below that for the reaction of HBsAg with anti-surface antigen antisera. This emphasizes that mere synthesis of peptides corresponding to part of the primary structure of a protein may not be adequate and a more sophisticated approach will be required to make peptides mimic the conformational determinants important for immunization.

INFLUENZA

Influenza is an important target for vaccine research as it remains a widespread epidemic disease in man. The production of a new vaccine will be particularly

difficult because of the variable antigenic structure of the virus. Vaccines made from inactivated or attenuated virus strains are not effective in preventing epidemics because new variants of the principal coat proteins, haemagglutinin (HA) and neuraminidase, arise in the population and enable the virus to escape the host immune defences. New vaccines will need to anticipate the appearance of virus variants or be constructed in such a way as to generate cross-strain protection.

The antigenic structure of the influenza virus coat protein HA has been thoroughly studied (*see* Webster *et al.*, 1982). The amino-acid sequences of many antigenic variants have been determined by protein-sequencing and gene-cloning techniques. This information is made more valuable by the successful determination of the three-dimensional structure of the protein (Wilson, Skehel and Wiley, 1981). HA has been synthesized in *E. coli* (Emtage *et al.*, 1980) and in mammalian cells (Gething and Sambrook, 1981) but most of the work in this field has been with synthetic peptides. Green *et al.* (1982) demonstrated that many peptides can generate antibodies which recognize haemagglutinin. A peptide corresponding to amino acids 91–108 has been used to immunize mice and to give them partial protection from challenge infection (Müller, Shapira and Arnon, 1982). This peptide was chosen as it is constant in sequence in many strains and so could avoid some of the problems associated with antigenic variation.

Another region of the HA molecule, amino-acid residues 1–11 of the HA2 part of HA, was selected for study because it was implicated in virus infection and could be a good target for immune defences (Scheid and Choppin, 1974). Atassi and Webster (1983) examined the antigenic properties of the synthetic peptides for this region for two strains of influenza virus. They found that nine out of 30 virus-neutralizing monoclonal antibodies bound completely or partly to the peptide. Antisera raised against the peptide could also bind to HA and intact virus but were not neutralizing. Furthermore, after injection the synthetic peptides failed to protect mice from infection. This puzzling observation shows that the immunological response must be assessed on the basis of immunoglobulin specificities and affinities. Possibly the peptide chosen in this study lacks part of the antibody-binding site or only partly mimics the virus conformation. A further complication arises from a lack of detailed understanding about the molecular and cellular mechanisms of virus neutralization. There are several stages in a virus life cycle that could be susceptible to antibody-mediated neutralization and they may require different vaccination strategies.

Bacterial subunit vaccines

In the past, commercially available subunit vaccines have consisted of partially purified culture-supernatant fractions or bacterial-cell-wall preparations. The molecular characterization of potent bacterial antigens capable of generating protective immunity has been achieved in only a few cases. This has limited the development of subunit vaccines which are enriched in the protective components and are antigenically well defined. However, great progress has been made in our understanding of the structure of the bacterial-cell surface

cases higher yields of the adhesion fimbriae (Kehoe *et al.*, 1981). However, in order to increase the range of protection offered by scour vaccines an antitoxin component is required.

ETEC strains express two classes of enterotoxin. The first class of enterotoxin, known as heat-labile toxins (LT-toxin), can be inactivated by heating at 60°C for 30 minutes. The second class, known as heat-stable toxins (ST-toxin), can be boiled and still retain activity. LT-toxins expressed by bovine and porcine isolates are identical proteins closely related to cholera toxin (Clements and Finkelstein, 1978; Gilligan, Brown and Robertson, 1983). The determinant for LT-toxin has been cloned into *E. coli* K12 (So, Dallas and Falkow, 1978). Like cholera toxin, LT-toxin consists of two subunits — an enzymatically active A polypeptide and a subunit consisting of five identical smaller B polypeptides. Using simple cloning procedures, strains have been constructed which express only the immunodominant, non-toxic B subunit at high levels. The LT-B determinant has been transformed into *E. coli* K12 strains expressing one or more adhesion fimbriae in order to make new strains suitable for vaccine production. At least two companies now market vaccines based on these constructions.

An ST vaccine component is not yet available. ST-toxin can be purified as a toxic peptide 18 amino acids in length. Most ETEC strains express related ST-toxins (Moseley *et al.*, 1983) although some strains express a less-well-characterized class of ST known as ST_{II}. Because of its small size ST is a poor immunogen. However ST can be made immunogenic by coupling it to other larger proteins which act as carriers (Frantz and Robertson, 1981). ST can be readily synthesized chemically from the individual amino acids and synthetic ST has been coupled chemically to purified LT-B subunit (Klipstein, Engert and Houghten, 1983a,b). This complex has been shown in experimental animals to give some degree of protection against both ST- and LT-toxin activity and could form the basis of future antitoxin vaccines (Klipstein *et al.*, 1983). Human ETEC strains express a number of different adhesion fimbriae and a human vaccine based on one type alone is not a viable proposition at present. However an ST- and LT-based vaccine might be suitable for use in both humans and animals as human ETEC strains express highly related LT- and ST-toxins.

CLONING ANTIGENS FROM FASTIDIOUS BACTERIAL PATHOGENS

Some bacterial pathogens are extremely difficult to cultivate outside their natural hosts and this makes it impossible or extremely tedious to prepare vaccines by conventional means. Organisms such as *Treponema pallidum* which causes human syphilis and *Mycobacterium leprae* which causes leprosy have never been grown *in vitro* and can be propagated only using laboratory animals. Even when fastidious organisms are cultivated on artificial media, great care must be taken to ensure that important protective antigens are expressed, as many pathogens attenuate rapidly upon passage *in vitro*. By employing gene-cloning techniques, important protective or potentially protective antigens can be expressed in more easily cultivated bacteria such as *E. coli*

or *Bacillus subtilis*. Cloning work has already begun on some fastidious pathogens and the initial results suggest that genes from most pathogenic bacteria can be directly expressed in *E. coli*, thus simplifying screening techniques.

T. pallidum, the cause of human syphilis, is a fastidious spirochaete which is extremely well adapted to surviving in mammalian hosts. To date the organism has not been cultivated for more than a few hours outside the host. For this reason insufficient quantities of the spirochaetes have been available to conduct fruitful genetic and biochemical studies and no syphilis vaccine has been developed. *T. pallidum* strains can be maintained by passage in rabbits and enough spirochaetes can be extracted from infected rabbit tissue to isolate sufficient DNA to generate gene banks of *T. pallidum* DNA in *E. coli* K12. Several groups have now succeeded in cloning and expressing *T. pallidum* genes in *E. coli* K12 (Walfield, Hanff and Lovett, 1982; Stamm *et al.*, 1983; van Emden *et al.*, 1983). The gene banks were screened to identify recombinants expressing specific *T. pallidum* antigens using convalescent sera obtained from rabbits or humans who had recovered from *T. pallidum* infections. Now for the first time large quantities of specific *T. pallidum* antigens, recognized by the immune system during natural infections, will be available. Initially, some of these antigens may find use as diagnostic reagents but eventually it is feasible that a syphilis vaccine could be developed.

Like *T. pallidum*, *Mycobacterium leprae* can be grown only in experimental animals, in this case the livers of armadillos. Cloning work has been initiated to try to isolate determinants for *M. leprae* surface proteins, some of which can be identified by monoclonal antibodies (Ivanyi *et al.*, 1983). The cloning of *M. leprae* antigens would be a major breakthrough in the development of diagnostic reagents for leprosy.

Bacteroides nodosus is a fastidious obligate anaerobic Gram-negative bacterium which is a major pathogen in sheep, causing ovine foot rot, a disease of great economic importance affecting sheep reared in damp climates. The organism is difficult to cultivate to high cell densities in laboratory media, especially in the fimbriated form which is essential for vaccine preparation (Stewart, 1978). Sheep can be protected against challenge by *B. nodosus* by vaccination. Current vaccines contain whole bacterial cells mixed with oil or alum adjuvants. The vaccines are reactogenic, often causing lesions at the site of injection. Experimental evidence suggests that surface-associated fimbriae expressed by *B. nodosus* are important protective antigens. These fimbriae form the basis of a *B. nodosus* serotyping system and many different serotypes have been identified, with up to eight serotypes being required for an effective vaccine. The fimbriae of serogroup A have been purified and the complete amino-acid sequence of the fimbrial subunit polypeptide has been determined (McKern *et al.*, 1983). More recently the determinant has been cloned into *E. coli* K12 and the fimbrial antigen was expressed efficiently within the recombinant cells. The cloning of other fimbrial serotypes should soon follow and an *E. coli* based vaccine could soon be available. Such a vaccine would be cheaper to produce than current vaccines.

Sequencing of *B. nodosus* fimbriae showed that the amino-terminal portion of the fimbrial subunit polypeptide was related to fimbriae subunit expressed by

Neisseria gonorrhoeae which causes gonorrhoea in humans. Like *B. nodosus*, *N. gonorrhoeae* can express many serologically distinct fimbrial types. Attempts have been made to use *N. gonorrhoeae* fimbriae preparations for vaccination in humans and experimental animals, although significant protection can be achieved only when both challenge strain and vaccine contain the homologous fimbrial type. Recent work using monoclonal antibodies has shown that although *N. gonorrhoeae* fimbriae are immunologically heterogeneous when tested with polyvalent sera, some monoclonal antibodies can react with all fimbrial types (Virji, Heckels and Watt, 1983). Thus they recognize a conserved region on the fimbriae which has been located on the amino-terminal region of the fimbrial subunit polypeptide. This region does not appear to be the immunodominant portion of the molecule when fimbriae are used to raise sera in experimental animals, although it is possible that peptides corresponding to this region could be made more immunogenic if they were presented correctly to the immune system.

GENE CLONING AS A TOOL FOR DEVELOPING LESS REACTOGENIC BACTERIAL VACCINES

Some vaccines can induce unpleasant side-reactions in vaccinates. Symptoms can take many forms, developing soon after vaccination or over a period of weeks following challenge, often (but not always) at the site of injection with parenteral vaccines. Reactogenicity can be caused by non-essential vaccine components or by adjuvants. Veterinary vaccines based on oil adjuvants are often particularly reactogenic, with severe lesions occurring at the site of vaccination. Gene cloning could be a valuable tool for developing less reactogenic vaccines. Reactogenicity should be reduced by increasing the purity and yield of specific protective antigens.

The current anthrax vaccine is prepared from alum-precipitated culture supernatants of *Bacillus anthracis*. The vaccine is reactogenic and a course of six injections followed by yearly boosters is required to achieve high-level protection. *B. anthracis* secretes three distinct proteins known as edema factor (EF), lethal factor (LF) and protective antigen (PA) which together constitute anthrax toxin (Vodkin and Leppla, 1983). PA is a binding protein required to present EF and LF to the eukaryotic target cells. For vaccine preparation *B. anthracis* is cultivated under conditions in which high levels of PA but barely detectable levels of EF and LF appear in the cultures. Vodkin and Leppla (1983) recently reported the cloning and expression of the PA from *B. anthracis* into *E. coli* K12 free of EF and LF. The recombinant strain could be modified in the future to form a new-generation anthrax vaccine.

Although whooping-cough vaccine is very effective it can induce side-effects in some young children who receive the vaccine. The current vaccine contains inactivated whole cells of *Bordetella pertussis*, the causative agent of whooping cough. Careful molecular analysis has recently led to the identification of important virulence factors such as pertussis toxin filamentous haemagglutinin and adenylate cyclase which could be important protective components of the vaccine. It is hoped that molecular analysis will be used in the future to develop an even safer whooping-cough vaccine.

Live attenuated vaccines

Individuals who have recovered from a natural microbial infection normally exhibit a high level of immunity to subsequent infection by the homologous microbe. Thus in the past it has been an attractive approach to use attenuated strains as vaccines. Such strains can establish limited infections in the host which are sufficient to induce protective immunity without causing significant clinical symptoms. Most live vaccine strains were derived either by extensively passaging fully virulent pathogens away from the natural host and selecting non-virulent derivatives or by isolating naturally occurring related non-pathogenic strains. Problems that can occur with this approach include the potential for attenuated microbes to revert to a fully virulent form and the loss of expression of important protective antigens following passage under artificial conditions. However, live attenuated vaccines have proved very effective and can offer the added attraction of inducing local immunity. Molecular and genetic techniques can be used to construct attenuated micro-organisms which have little or no potential to revert to a virulent form. Furthermore, it is possible to move the genetic information for a protective antigen of one pathogen into an attenuated strain derived from a second pathogen, thus constructing a potentially bivalent vaccine strain.

LIVE ATTENUATED ENTERIC BACTERIAL VACCINES

It has long been recognized that parenteral vaccination, either with live or inactivated vaccines, is an inefficient method for inducing long-lasting protective immunity against many bacterial enteropathogens. The stimulation of the local gut immune response which occurs during natural infections is believed to be important in achieving good protection. Thus there is a requirement for attenuated strains suitable for use as live oral vaccines. Traditional approaches to selecting such strains have not been successful. However, several recent attempts have been made to construct suitable strains using genetic manipulation.

Salmonella *vaccines*

Most currently available *Salmonella* vaccines are parenteral vaccines which consist of attenuated live cells or inactivated cells. *Salmonella typhi* is the causative agent of human typhoid. The current parenteral vaccines can cause several side-reactions and there is a demand for a live oral typhoid vaccine. *S. typhi* is principally a human pathogen and is extremely infectious and virulent. Although the lack of suitable animal model systems inhibited work on *S. typhi*, a well-characterized mouse model exists for the related pathogen *S. typhimurium*. Work carried out using rough mutants of *S. typhimurium* in the mouse-model system pointed to a potential approach to live vaccine. Rough mutants are defective in the synthesis of surface-associated LPS. Such mutants exhibit reduced virulence compared with parental smooth strains. Germanier

(1970) compared the virulence and vaccine potential of various rough mutants of *S. typhimurium* defective in different stages of LPS synthesis and found that *gal* E mutants induced significantly higher levels of protective immunity in mice than other rough strains. The *gal* E gene encodes for an enzyme which is required for galactose metabolism. Although *gal* E mutants are normally rough, they become smooth when grown on medium containing galactose. However, prolonged contact with galactose results in cell death and lysis. The *gal* E mutants can be propagated efficiently on medium lacking galactose. When fed to animals, the strains metabolize the galactose available in the host gut lumen and tissues and die after a few cycles of replication. The limited period of viability is the key to its use in delivering antigens to the host immune system.

Germanier and Furer (1975) went on to construct a *gal* E derivative of *S. typhi* using nitrosoguanidine mutagenesis. The strain did not revert to a galactose metabolizer at a detectable frequency. The strain, known as *S. typhi* 21a, was put forward as a potential live oral-vaccine strain for human use. The results of volunteer trials were encouraging and a large-scale field trial was carried out using children in Egypt over a three-year period. The vaccine showed a high degree of efficiency when the children were given three oral doses of the vaccine (Wadhan *et al.*, 1982). A second field trial in Santiago, Chile, an area with a much higher natural incidence of endemic typhoid, was less conclusive (*see* Levine *et al.*, 1983b for a discussion of these data). In this trial less than three doses of vaccine were used and the efficacy was lower than that found in Egypt. Nevertheless *S. typhi* 21a shows promise as a live oral vaccine and more extensive trials are currently in progress.

Salmonellosis is an economically important disease in domestic animals. Current vaccines are avirulent rough strains of *S. typhimurium* and *S. cholera-suis* administered parenterally. However, unlike *gal* E mutants, these strains do not synthesize LPS (an important protective antigen). Virulence studies carried out on *Salmonella* indicated that certain auxotrophic mutations could reduce the virulence of pathogenic strains. Auxotrophs that required aromatic compounds which are normally synthesized via a common metabolic pathway showed reduced virulence. Such auxotrophs carried mutations in the *aro* genes (Hoiseth and Stocker, 1981). These compounds are not freely available in mammalian tissues and strains auxotrophic for these compounds grow poorly in the mammalian host. Stocker, Hoiseth and Smith (1983) used *in vivo* genetic engineering to construct strains of *S. typhimurium* carrying transposon Tn10 insertions in the *aro* A gene. Tn10 encodes for tetracycline resistance and insertion of Tn10 into the coding sequence of a gene will lead to inactivation. However Tn10 can generate deletions at the site of insertion and by selecting for tetracycline-sensitive derivatives it was possible to select for mutants of *S. typhimurium* carrying deletions in the *aro* gene. Transduction was used to introduce the mutation into calf-virulent strains of *S. dublin* and *S. typhimurium*. These strains were then used, in single doses, to vaccinate calves by the oral route. The *aro* A derivative induced a significant level of protection in calves against virulent challenge strains and the efficacy of the vaccine was greater than that of the parenteral vaccine (Robertsson *et al.*, 1983).

Cholera vaccines

A different approach has been used to construct attenuated derivatives of *Vibrio cholerae*. Cholera is a non-invasive infection primarily affecting the small bowel. A major virulence factor of *V. cholerae* is cholera toxin which, by activating adenylate cyclase, the enzyme intimately connected with secretory processes of the intestinal mucosal cells, is responsible for the massive fluid loss induced in victims of the disease. Cholera toxin (the holotoxin) is composed of two subunits. The A subunit is the portion of the molecule which affects adenylate cyclase and consists of a single 27 000 dalton polypeptide. The B subunit consists of five identical 11 600 dalton polypeptides responsible for binding the toxin to the mucosal epithelial-cell surface and delivering the A subunit to the target-cell membrane. The B subunit is the immunodominant portion of the molecule and antisera against the B subunit can protect against the functional cholera toxin.

Mutants of *V. cholerae* defective in cholera-toxin production show reduced levels of virulence in model systems. Strains have been isolated which synthesize inactivated cholera toxin. As the A subunit of cholera toxin is responsible for diarrhoeal symptoms a *V. cholerae* strain expressing only the B subunit would be a candidate as a live oral vaccine. Honda and Finkelstein (1979) used nitrosoguanidine mutagenesis to select a mutant of *V. cholerae* expressing high levels of the B subunit but no detectable levels of the A subunit. They named the strain Texas Star-SR and showed that it had reduced virulence in animal model systems (Honda and Finkelstein, 1979). The strain was further tested in human volunteers and was found to have a high efficiency following a single- or double-dose oral challenge. However some of the volunteers challenged with Texas Star-SR produced some loose stools although the level of diarrhoea was much milder than that found with fully virulent *V. cholerae* strains. Careful analysis of the stools of individuals who had ingested live Texas Star-SR organisms failed to detect revertants which were able to produce cholera holotoxin (Levine *et al.*, 1983a).

Because the genetic lesion carried in Texas Star-SR was ill defined, other groups set out to produce mutants of *V. cholerae* carrying more defined mutations within the toxin A subunit gene. The first step was to clone the toxin genes from the *V. cholerae* chromosome using DNA probes encoding the related *E. coli* heat-labile toxin determinant (Mekalanos *et al.*, 1983). The toxin gene was cloned into plasmid vectors and shown to be expressed in *E. coli*. The determinant was then subjected to mapping and DNA sequence analysis. Once the coding region for the A and B subunits had been shown to map in a single operon, specific deletion and insertion mutants were constructed within the cistron encoding the A subunit. The deletion mutants were then reintroduced into *V. cholerae* and genetic manipulation involving DNA homology was used to replace chromosomal copies of the cholera toxin determinant with the deletion mutants. Thus attenuated mutants of *V. cholerae* were constructed which carried well-characterized lesions. The presence of the lesions in the *V. cholerae* chromosome was confirmed using Southern blotting analysis. Volunteer studies carried out with these engineered strains produced

similar results to those obtained with Texas Star-SR. Some of the volunteers produced a few loose stools although the efficacy of the vaccine was high. Attempts are currently under way to attenuate *V. cholerae* toxin-defective strains further in order to prevent all diarrhoeal symptoms (Kaper *et al.*, 1984).

LIVE ORAL VACCINES AS CARRIERS FOR OTHER ANTIGENS

As attempts to produce a new generation of live oral vaccines against enteric infections are showing great promise it may be possible to construct strains which protect against more than one enteric pathogen. Formal *et al.* (1981) introduced a 120 megadalton plasmid from *Shigella sonnei* into the attenuated *S. typhi gal* E mutant Ty21a. This plasmid encodes for *S. sonnei* O antigens and possibly other important *Shigella* surface antigens. Tests with animals and human volunteers have shown that this vaccine strain causes no adverse reactions even when ingested in high doses (Levine *et al.*, 1983b). It is an attractive idea to use attenuated bacterial strains to deliver other protective antigens to the gut in order to stimulate the local secretory immune system. It will be interesting to see if this approach is successful in inducing protective immunity in humans or domestic animals.

LIVE ATTENUATED VIRAL VACCINES

The potential advantages of live vaccines in cost and efficacy have been a guiding force in viral-vaccine research over the past decades. Many examples of successful vaccination with attenuated viral strains now exist. However, the development of attenuated strains has traditionally been an uncertain process. It has been observed that passaging viruses in unnatural hosts or tissue culture systems often results in an altered strain unable to produce a virulent infection in the natural host, yet still capable of replication. Attenuated vaccines have been used even though the basis for avirulence is unknown and the possibility of reversion to wild type remains. Considerable effort is needed in the safety testing of such vaccines to minimize the risks of actually spreading infection through vaccination with live viruses.

Unfortunately the modified viral strains that result from the above techniques may be altered in ways that affect their use as immunogens. Cold-adapted mutants of respiratory syncytial virus, which are unable to grow at normal body temperatures but can be cultivated in laboratory conditions, can be simply administered into the respiratory tract. Unfortunately these vaccines have so far proved unsatisfactory due to poor immunogenicity, excess reactogenicity and problems associated with reversion to wild type and recombination with virus strains in the environment.

The process of generating attenuated strains can be greatly improved for those viruses which contain segmented genomes. The desirable growth and avirulent characteristics of a mutant strain can be easily combined with the antigenic components of new variants in the field. Cold-adapted influenza

strains which have been effective in preventing natural challenge can be used as master strains to transfer speedily and effectively the phenotype of attenuation during reassortment with wild-type viruses. This strategy is also possible with another virus with a segmented genome, rotavirus, which is a major cause of diarrhoea.

Genetic engineering techniques can assist in the production of attenuated strains in two ways. Firstly, the increased knowledge of the molecular basis of virulence and antigenicity enables a better assessment of the safety and suitability of vaccine strains already in use. Secondly, recombinant DNA technology can be used deliberately to construct defective viruses with desirable properties. For viruses with double-stranded DNA genomes or where double-stranded DNA is a part of the replication cycle, standard gene-cloning procedures may be applicable to introduce deletions, insertions or point mutations into any specific portion of the genome at will. For viruses with RNA genomes this would not be possible. However, it has been reported that full-length complementary DNA clones of poliovirus are infectious in mammalian cells and result in the production of intact virus (Racaniello and Baltimore, 1981). This opens the door for a detailed analysis of poliovirus virulence and, it is hoped, the construction of effective, attenuated vaccine strains incapable of reverting to virulence. Attenuated virus vaccines seem less feasible for some other viruses such as herpes and hepatitis B where there is latent or recurrent infection, in some cases implicated with the integration of the virus into the host genome. For such viruses, proving the safety of an attenuated strain may be extremely difficult.

An additional problem with some live vaccines that are parenterally administered is that resident antibody of maternal or acquired origin may prevent virus growth and subsequent further stimulation of the immune response. Any live vaccine can thus suffer from the efficacy of the immune system in stopping virus replication. Maternal antibody persists in infants for varying periods yet it may be necessary to administer some vaccines early in life. It is harder to predict when adults may have antibody as a result of natural infection which complicates field trials, so extensive immunological screening may be necessary.

LIVE HETEROLOGOUS VIRAL VACCINES

Despite the success of a number of live virus vaccines there are many viruses where such an approach has not succeeded because it is not possible to combine all of the desirable features in a single organism. Because of genome instability or recombination in the wild it may never be possible to prevent reversion to virulence. Similarly, it would be hard to produce completely safe live vaccines from viruses which are capable of integrating into the host genome. In both cases a live vaccine may be achieved only by incorporating the important antigenic determinants into a totally separate non-pathogenic, self-replicating virus. Such heterologous viruses have now been constructed and the results are already very promising.

Considerable progress has been made with the use of vaccinia as a carrier for

foreign antigenic determinants (*see* Chapter 14 of this volume). Several genes from other viruses have now been inserted into the large vaccinia genome at places that do not disrupt any essential vaccinia gene functions. The principle of the method is that foreign DNA flanked by vaccinia DNA sequences could be introduced by transfection procedures into virus-infected cells. There is a site-specific *in vivo* recombination between the vaccinia sequences on the foreign DNA and the homologous sequences on replicating viral DNA allowing insertion of foreign DNA into infectious viral progeny. The recombinant vaccinia viruses can then be identified and isolated in pure form. It is further desirable that the foreign gene is placed downstream from a suitable vaccinia promoter to ensure that adequate levels of expression of antigenic sequences occur in the heterologous host.

Smith, Mackett and Moss (1983) inserted a 1.35 kilobase pair *Bam* HI fragment containing the HBsAg gene into the vaccinia genome under the control of a vaccinia early promoter. High levels of surface antigen were expressed, exceeding that produced by hepatoma cell lines. Surface antigen particles were excreted from cells infected with vaccinia-virus recombinants with properties similar to the particles isolated from hepatoma cells. Vaccinia recombinants could be used as a source of soluble antigen but the main intention is to inoculate intradermally with live virus where the antigen can be amplified during replication and present the host's immunological system with sufficient antigenic mass. Rabbits respond to live vaccinia recombinants with very high levels of anti-hepatitis antibodies. Paoletti *et al.* (1984) also report the successful construction and use of vaccinia recombinants containing HBsAg.

Vaccinia-virus recombinants carrying influenza virus haemagglutinin (Panicali *et al.*, 1983) and herpes simplex virus glycoprotein D (Paoletti *et al.*, 1984) have also been reported. For both cases the hybrid vaccinia was inoculated into rabbits and virus-neutralizing antibodies were detectable. More importantly, immunization of mice with the vaccinia recombinant expressing herpes simplex virus glycoprotein D gave complete protection on subsequent challenge with lethal doses of live herpes virus (Paoletti *et al.*, 1984). The vaccinia system thus seems well suited to the expression of viral membrane protein antigens. This system may not be so well suited to the production of virus antigens which form a protein coat without membranes.

Even when vaccinia can be successfully used in the laboratory the use of this virus for human vaccinations is controversial. Vaccinia itself can have complications, with adverse reactions occurring at a frequency of one case per 1000 (Behbehani, 1983). Vaccinia carrying foreign surface antigens could acquire new trophisms and pathogenicity which would have to be carefully examined. Nevertheless the advantages of the system are so overwhelming that the effort is certainly worth while.

Having used vaccinia to establish the principle of heterologous live vaccines, it should be possible to develop alternative virus carriers. The requirements for such a virus are that it should replicate in the target host, the host immune system should not have antibodies to the virus that interfere with its replication, and the virus genome must have excess capacity to incorporate foreign antigenic coding sequences. Dulbecco (1983) describes recombinants of

and extracellular products and this is leading to a demand for subunit vaccines to replace old whole-cell vaccines. Even in the case of existing subunit vaccines such as diphtheria vaccine there is an increasing demand for a purer product simply because it is now possible to produce one. Genetic manipulation can contribute to the development of bacterial subunit vaccines in several ways. Together with immunological techniques, gene cloning can be used to identify and later modify potential protective bacterial antigens. The genetic determinants for protective antigens can be moved from fastidious pathogens into well-characterized hosts such as *E. coli* K12, thus simplifying vaccine production. Increased yields of antigen can be prepared in the absence of the risks associated with handling dangerous pathogens. Cloned determinants can be modified and re-introduced back into the parental pathogen to construct attenuated strains or strains expressing higher yields of antigen or toxoid. Although in many cases work is in relatively early stages several examples of possible future bacterial subunit vaccines will be discussed.

E. COLI SCOUR VACCINES

Scouring is an economically important diarrhoeal disease occurring predominantly during the first few days after birth in piglets and calves. Susceptible offspring can be passively protected when they suckle the colostrum and milk of immune mothers. Although several pathogens can contribute to scouring, one of the most common causes is enterotoxigenic *E. coli* (ETEC) which colonizes the small bowel of the animals and secretes one or more different enterotoxins (Smith and Linggood, 1971). ETEC strains can colonize the small bowel because they possess surface-associated fimbriae which attach the bacteria to the enterocytes lining the bowel lumen. These fimbriae are potent immunogens which are referred to as adhesion antigens (Gaastra and de Graaf, 1982). ETEC strains express several immunologically distinct fimbriae although individual strains normally express only one type. Porcine isolates express either K88, 987P or (more rarely) K99 antigens, whereas bovine isolates express K99 and F41 antigen. However, some porcine and bovine ETEC strains possess none of these fimbriae.

Early vaccines consisted of either whole cells expressing fimbriae or acellular fractions enriched in the fimbriae responsible for adhesion. High levels of protective immunity can be induced using fimbrial vaccines although they will protect against only those strains expressing the homologous fimbrial antigen. No toxin component was included in these vaccines.

Vaccines prepared from ETEC strains which do not produce toxin can cause adverse reactions in the recipients because of the high levels of lipopolysaccharide (LPS) and capsular material possessed by wild strains. Attempts were made to improve vaccine strains by cloning the adhesion-fimbriae determinants into *E. coli* K12 which expresses a rough LPS and causes fewer adverse reactions. The determinants for the K88, K99 and 987P antigens have been cloned on to multiple copy vectors in *E. coli* K12 (van Emden *et al.*, 1980; Shipley, Dougan and Falkow, 1981). The cloned genes can be used to construct strains expressing more than one fimbrial antigen (Kehoe *et al.*, 1982) and in some

adenovirus type 2 which carry amino-acid sequences from the poliovirus capsid proteins. Inoculation of this live virus into rabbits results in antisera capable of recognizing poliovirus. Whether this would function as an effective polio vaccine is not clear.

A live heterologous vaccine could also be constructed with a 'carrier' virus which cannot replicate in the host species. Such heterologous viral vaccines would be useful where the virus carrying a foreign antigenic determinant becomes a better immunogen than the purified antigen alone. The use of bacteriophage lambda carrying vesicular stomatitis virus (VSV) G protein is described by Dulbecco (1983) (*see also* Chapter 14 of this volume). The VSV antigenic site is exposed to the outside of the phage coat and is accessible to the immune system of a vaccinated animal. The use of viruses to carry antigens that can more effectively stimulate the immune system may be a solution to the low immunogenicity reported for most subunit vaccines.

Vaccines against parasitic infections

The development of vaccines against parasitic infections is a rapidly expanding area of research and only a brief overview can be presented here. Parasites present a greater challenge to vaccine design than do most bacteria and viruses and to date there has been only minor success in this field (Cox, 1982). This is largely due to the complexity of the parasite life cycles and the difficulty of cultivating the organisms *in vitro*. Parasites may also exhibit other features which could complicate the immunoprophylactic approach: they can have intracellular habitats, extreme antigenic variation and can often immunosuppress the host. Naturally acquired immunity to parasitic diseases is rarely complete and the immune system can usually only suppress parasite numbers rather than achieve a total 'sterile' immunity. It is hoped that new technologies can produce effective vaccines even where the disease itself cannot confer immunity but this goal has yet to be achieved. Vaccines for helminth diseases such as schistosomiasis are particularly difficult (Hoffman, Phillips and Cook, 1981). There is only circumstantial evidence for immunity which must be aimed at preventing establishment of the worms. Vaccines which decrease the longevity or fecundity of the worms would not stop infection but could reduce the spread of the disease to others.

The range of protozoan diseases for which vaccination is desirable is not vast but they all present special problems. For example, the African trypanosomes which cause sleeping sickness have been much studied and the details of the molecular mechanisms by which they change their surface coat protein are now becoming clear (Parsons, Nelson and Agabian, 1984). Although the coat proteins are highly immunogenic the number of immunological variants is probably greater than 100 and makes vaccination against all serotypes difficult if not impossible. In contrast, *Trypanosoma cruzi*, the cause of Chagas' disease in the Americas, does not exhibit antigenic variation on this scale and some success has been reported for immunization with defined antigens (Snary, 1983).

MALARIA

Many research groups have carried out research on vaccines for malaria, perhaps the most important parasitic disease of man. The *Plasmodium* parasites have a complex life cycle and immunization could be directed at any of the principal stages (sporozoite, erythrocytic schizont, merozoite and gametocyte).

Unfortunately, immunization against one stage of the life cycle does not extend to others and an effective vaccine may need to incorporate antigens from more than one stage (Cohen, 1982). Killed organisms from all of these stages have been tested successfully as experimental vaccines but more recent work has concentrated on obtaining purified antigens using monoclonal antibodies. Having identified protective antigens, gene-cloning technology would allow the production of protein antigen in vast quantities in *E. coli*.

The gene for the highly immunogenic surface protein of the sporozoite stage of the monkey malarial parasite *Plasmodium knowlesi* has been cloned and shown to have an unusual structure (Godson *et al.*, 1983) with at least one-third of the protein being composed of several repeats of a 12-amino-acid sequence. A synthetic dodecapeptide corresponding to this repeat unit behaves in an identical manner to the native protein in an immunoradiometric assay and is a potential vaccine immunogen. This work will need to be extended to the human malarial parasites.

Recombinant-DNA techniques are also being successfully applied to the merozoite stage of malaria. Although the sporozoite stage has only a single dominant surface antigen the blood stages of *P. falciparum* contain a plethora of natural immunogens, more than one of which are probably protective. It has been necessary to analyse several purified antigen preparations to identify candidates for immunization and gene-cloning experiments (Freeman and Holder, 1983). Several groups have recently reported the successful cloning of antigens from merozoites and some of these appear to contain repeating determinants as found with the sporozoite antigen. The significance of this observation is not yet clear.

A different approach has been followed by Kemp *et al.* (1983) who constructed malarial cDNA gene banks in *E. coli* with a specialized expression vector. They identified recombinants producing proteins which react with antibodies from immune humans. Proteins prepared from these selected recombinants can be thought of as 'monoclonal antigens' which will be useful in subsequent studies of malarial immunity.

Rapid though progress has been, there are still formidable problems facing the development of a malarial vaccine, both in terms of identifying and producing potent immunogens and in devising vaccination strategies that give long-lasting protection, especially where infection is normally acquired early in life as it is with malaria. It is likely that progress in this direction will be hindered by conflicting commercial and political interests. Although the potential market for a malarial vaccine is vast, with over 200 million individuals infected at any one time, it is primarily a disease of developing countries which will not be able to support an expensive high-technology vaccine.

Summary

The ability to move genetic determinants between species using *in vitro* gene-manipulation techniques has opened up new approaches to vaccine development. This has rapidly grown into an exciting area of research in both academic and industrial laboratories. There are numerous scientific challenges which require multidisciplinary teams to solve problems in creating new immunogens. This has challenged our existing knowledge about protein structure and conformation, microbial pathogenicity and the immune system.

Recombinant-DNA techniques are invaluable as tools of analysis and antigen production. The surface of micro-organisms can also be minutely explored with the use of synthetic peptides and monoclonal antibodies. Nevertheless, these new technologies do not allow us to circumvent the need for detailed understanding of pathogens and the disease process. What is apparent from the work carried out so far is that there are few easy answers to vaccine development and it is not realistic to expect rapid solutions to these problems.

As there are many potential targets for constructing novel vaccines for both human and animal diseases, it is helpful to establish some priorities. There is a tendency to look at the existing effective vaccines and simply direct research at producing them more economically or with enhanced safety and stability. The advantage of this approach is that considerable background work will have already been carried out establishing the basis for the application of recombinant DNA techniques. However, this can also lead to conflicts (often within the same institute or company) between the new and old technologies. This could be to the detriment of the new technologies which are still only partly developed and may not be good enough yet to compete with existing vaccines in cost or efficacy.

The more ambitious, and eventually more rewarding, approach is to attempt to develop new vaccines where none had existed before. There is a vast untapped market, especially in the parasitic diseases, but the scientific problems may be considerable and much more background work is likely to be necessary. Indeed, most of the work in this area is more accurately referred to as basic research rather than vaccine development as totally new, effective vaccines are still some way off.

Having directed research towards a specific organism or disease there are still many options available as to the scientific strategy to adopt. As discussed in this review it may be possible to consider subunits, synthetic antigens and live (attenuated or heterologous) organisms as possible vaccines. Where all options cannot be simultaneously pursued then again some priorities will be needed. Unfortunately it is too early to draw conclusions about the validity of each strategy as few comparative studies have been carried out and further technological advances could alter the picture dramatically. It is also premature to discuss the product costs associated with the new technologies as the dosages and efficacy have yet to be worked out. In addition, the regulatory authorities may demand extensive purification, safety and stability testing to be carried out, which may add considerably to the final product cost.

For mass immunization to eradicate the major diseases of man, the consumer acceptance of the new vaccines will be important. The development of more oral vaccines is particularly important in this regard but other new strategies to immunization should also be explored. The use of live vaccines, while solving many of these problems, remains controversial when recombinant strains are involved. However, with the present rate of progress in vaccine research it seems clear that most of these problems will be solved, resulting in a wide range of new and important vaccines by the end of the century.

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References

ADA, G.L. (1982). Vaccines for the future. *Australian Journal of Experimental Biology and Medical Science* **60**, 549–569.

ANDERSON, R.M. AND MAY, R.M. (1982). Directly transmitted infectious diseases: control by vaccination. *Science* **215**, 1053–1060.

ARNON, R., MARON, E., SELA, M. AND ANFINSEN, C.B. (1971). Antibodies reactive with native lysozyme elicited by a completely synthetic antigen. *Proceedings of the National Academy of Sciences of the United States of America* **68**, 1450–1455.

ATASSI, M.Z. (1977). The complete antigenic structure of myoglobin: approaches and conclusions for antigenic structure of proteins. In *Immunochemistry of Proteins* (M.Z. Atassi, Ed.), volume 2, pp. 77–176. Plenum Press, New York.

ATASSI, M.Z. AND HABEEB, A.F.S.A. (1977). The antigenic structure of hen egg-white lysozyme: a model for disulfide-containing proteins. In *Immunochemistry of Proteins* (M.Z. Atassi, Ed.), volume 2, pp. 177–264. Plenum Press, New York.

ATASSI, M.Z. AND WEBSTER, R.G. (1983). Localization, synthesis and activity of an antigenic site on influenza virus hemagglutinin. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 840–844.

AUSTRIAN, R., DOUGLAS, R.M., SCHIFFMAN, G., COETZEE, A.M., KOORNHOF, H.J., HAYDEN-SMITH, S. AND REID, R.D.W. (1976). Prevention of pneumococcal pneumonia by vaccination. *Transactions of the Association of American Physicians* **89**, 184–190.

BACHRACH, H.L., MOORE, D.M., MCKERCHER, P.D. AND POLATNICK, J. (1975). Immune and antibody responses to an isolated capsid protein of foot-and-mouth disease virus. *Journal of Immunology* **115**, 1636–1641.

BECK, E., FEIL, G. AND STROHMAIER, K. (1983). The molecular basis of the antigenic variation of foot-and-mouth disease virus. *EMBO Journal* **2**, 555–559.

BEHBEHANI, A.M. (1983). The smallpox story: life and death of an old disease. *Microbiological Reviews* **47**, 455–509.

BHATNAGAR, P.K., PAPAS, E., BLUM, H.E., MILICH, D.R., NITECKI, D., KARELS, M.J. AND VYAS, G.N. (1982). Immune response to synthetic peptide analogues of hepatitis B surface antigen for the *a* determinant. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 4400–4404.

BITTLE, J.L., HOUGHTEN, R.A., ALEXANDER, H., SHINNICK, T.M., SUTCLIFFE, J.G., LERNER, R.A., ROWLANDS, D.J. AND BROWN, F. (1982). Protection against FMDV by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. *Nature* **298**, 30–34.

Bomford, R. (1983). Basic immunology and vaccination. In *Pharmacological Basis of Large Animal Medicine* (J.A. Bogan, P. Lees and A.T. Yoxall, Eds), pp. 145–158, Blackwell Scientific Publications, Oxford.

Bomford, R. (1984). Immunological adjuvants. In *Textbook of Immunopharmacology* (M. Dale and J. Foremann, Eds), pp. 340–346. Blackwell Scientific Publications, Oxford.

Boothroyd, J.C., Highfield, P.E., Cross, G.A.M., Rowlands, D.J., Lowe, P.A., Brown, F. and Harris, T.J.R. (1981). Molecular cloning of foot-and-mouth disease virus genome and the nucleotide sequences in the structural protein genes. *Nature* **290**, 800–802.

Broome, S. and Gilbert, W. (1978). Immunological screening method to detect specific translation products. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 2746–2750.

Burrell, C.J., Mackay, P., Greenaway, P.J., Hofschneider, P.H. and Murray, K. (1979). Expression in *Escherichia coli* of hepatitis B virus DNA sequence cloned in plasmid pBR322. *Nature* **279,** 43–47.

Chedid, L.A., Parant, M.A., Audibert, F.M., Riveau, G.J., Parant, F.J., Lederer, E., Choay, J.P. and Le Francier, P.L. (1982). Biological activity of a new synthetic muramyl peptide adjuvant devoid of pyrogenicity. *Infection and Immunity* **35**, 417–424.

Cheung, A.K. and Küpper, H. (1984). Biotechnological approach to a new foot-and-mouth disease virus vaccine. In *Biotechnology and Genetic Engineering Reviews* (G.E. Russell, Ed.), volume 1, pp. 223–259, Intercept, Newcastle upon Tyne.

Clarke, B.E., Carroll, A.P., Rowlands, D.J., Nicholson, B.H., Houghten, R.A., Lerner, R.A. and Brown, F. (1983). Synthetic peptides mimic subtype specificity of foot-and-mouth disease virus. *FEBS Letters* **157**, 261–264.

Clements, J.D. and Finkelstein, R.A. (1978). Demonstration of shared and unique immunological determinants in enterotoxins from *Vibrio cholerae* and *Escherichia coli*. *Infection and Immunity* **22**, 709–713.

Cohen, S. (1982). Progress in malaria vaccine development. In *Parasites — Their World and Ours* (D.F. Mettrick and S.S. Desser, Eds), pp. 408–411. Elsevier Biomedical Press, Amsterdam.

Cox, F.E.G. (1982). Vaccines for parasitic diseases. In *Parasites — Their World and Ours* (D.F. Mettrick and S.S. Desser, Eds), pp. 395–404. Elsevier Biomedical Press, Amsterdam.

Creese, A.L., Sriyabbaya, N., Casabal, G. and Wiseso, G. (1982). Cost-effectiveness appraisal of immunization programmes. *Bulletin of the World Health Organization* **60**, 621–632.

de Boer, H.A., Comstock, L.J., Yansura, D.G. and Heynecker, H.L. (1982). Construction of a tandem trp-lac promoter and a hybrid trp-lac promoter for efficient and controlled expression of the human growth hormone gene in *Escherichia coli*. In *Promoter Structure and Function* (R.L. Rodriguez and M.J. Chamberlain, Eds). Praegar Publishers, New York.

Dreesman, G.R., Sanchez, I., Ionescu-Matiu, J.T., Sparrow, H.R., Six, D.L., Peterson, D.L., Hollinger, F.B. and Melnick, J.L. (1982). Antibody to hepatitis B surface antigen after a single inoculation of uncoupled synthetic HBsAg peptides. *Nature* **295**, 158–160.

Dubois, M.F., Pourcel, C., Roussets, S., Chany, C. and Tiollais, P. (1980). Excretion of hepatitis B surface antigen particles from mouse cells transformed with cloned viral DNA. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 4549–4553.

Dulbecco, R. (1983). *Viruses with Recombinant Surface Proteins.* International Patent Application Number PCT/US83/00015.

Edelman, R. (1980). Vaccine adjuvants. *Reviews of Infectious Diseases* **2**, 370–383.

Efstratiadis, A. and Villa-Komaroff, L. (1979). Cloning of double-stranded DNA. In *Genetic Engineering* (J.K. Setlow and A. Hollaender, Eds), volume 1, pp. 15–36. Plenum Press, New York.

EMINI, E.A., JAMESON, B.A. AND WIMMER, E. (1983). Priming for an induction of anti-poliovirus neutralizing antibodies by synthetic peptides. *Nature* **304**, 699–703.

EMINI, E.A., JAMESON, B.A., LEWIS, A.J., LARSEN, G.R. AND WIMMER, E. (1982). Poliovirus neutralization epitopes: analysis and localization with neutralizing monoclonal antibodies. *Journal of Virology* **43**, 997–1005.

EMTAGE, J.S., TACON, W.C.A., CATLIN, G.H., JENKINS, B., PORTER, A.G. AND CAREY, N.H. (1980). Influenza antigenic determinants are expressed from haemagglutinin genes cloned in *Escherichia coli*. *Nature* **283**, 171–174.

EVANS, D.M.A., MINOR, P.D., SCHILD, G.S. AND ALMOND, J.W. (1983). Critical role of an eight-amino-acid sequence of VP1 in neutralization of poliovirus type 3. *Nature* **304**, 459–462.

FORMAL, S.B., BARON, L.S., KOPECKO, D.J., WASHINGTON, O., POWELL, C. AND LIFE, C.A. (1981). Construction of a potential bivalent vaccine strain: introduction of *Shigella sonnei* form 1 antigen genes into *gal* E *Salmonella typhi* Ty21a typhoid vaccine strain. *Infection and Immunity* **34**, 746–750.

FRANTZ, J.C. AND ROBERTSON, D.C. (1981). Immunological properties of *Escherichia coli* heat-stable enterotoxins: development of a radioimmunoassay specific for heat-stable enterotoxins with suckling mouse activity. *Infection and Immunity* **33**, 193–198.

FREEMAN, R. AND HOLDER, A.A. (1983). Surface antigens of malarial merozoites. *Journal of Experimental Medicine* **158**, 1647–1653.

FUNDENBERG, H.H., STITES, D.P., CALDWELL, J.L. AND WELLS, J.V. (1978). *Basic and Clinical Immunology*, 2nd edition. Lange Medical Publications, Los Altos, California.

GAASTRA, W. AND DE GRAAF, F.K. (1982). Host-specific fimbrial adhesins of non-invasive enterotoxigenic *Escherichia coli* strains. *Microbiological Reviews* **46**, 129–161.

GERMANIER, R. (1970). Immunity in experimental salmonellosis. I. Protection induced by rough mutants of *Salmonella typhimurium*. *Infection and Immunity* **2**, 309–315.

GERMANIER, R. AND FURER, E. (1975). Isolation and characterization of *gal* E mutant Ty21a of *Salmonella typhi*: a candidate strain for a live, oral typhoid vaccine. *Journal of Infectious Diseases* **131**, 553–558.

GETHING, M.J. AND SAMBROOK, J. (1981). Cell-surface expression of influenza haemagglutinin from a cloned DNA copy of the RNA gene. *Nature* **293**, 620–625.

GILLIGAN, P.H., BROWN, J.C. AND ROBERTSON, D.C. (1983). Immunological relationship between cholera toxin and *Escherichia coli* heat-labile enterotoxin. *Infection and Immunity* **42**, 683–691.

GODSON, G.N., ELLIS, J., SVEC, P., SCHLESINGER, D.H. AND NUSSENZWEIG, V. (1983). Identification and chemical synthesis of a tandemly repeated immunogenic region of *Plasmodium knowlesi* circumsporozoite protein. *Nature* **305**, 29–33.

GREEN, N., ALEXANDER, H., OLSON, A., ALEXANDER, S., SHINNICK, T.M., SUTCLIFFE, J.G. AND LERNER, R.A. (1982). Immunogenic structure of the influenza virus haemagglutinin. *Cell* **28**, 477–487.

GUARANTE, L., ROBERTS, T.M. AND PTASHNE, M. (1980). A technique for expressing eukaryotic genes in bacteria. *Science* **209**, 1428–1430.

HENNING, U., COLE, S.T., BREMER, E., HINDENNACH, I. AND SCHALLER, H. (1983). Gene fusion using the *omp* A gene coding for a major outer-membrane protein of *Escherichia coli* K12. *European Journal of Biochemistry* **136**, 233–240.

HOFFMAN, D.B., PHILLIPS, S.M. AND COOK, J.A. (1981). Vaccine development for schistosomiasis — report of a workshop. *American Journal of Tropical Medicine and Hygiene* **30**, 1247–1251.

HOISETH, S.K. AND STOCKER, B.A.D. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**, 238.

HONDA, T. AND FINKELSTEIN, R.A. (1979). Selection and characterization of a *Vibrio cholerae* mutant lacking the A (ADP-ribosylating) portion of the cholera enterotoxin. *Proceedings of the National Academy of Science of the United States of America* **76**, 2052–2056.

HONDA, T. AND FINKELSTEIN, R. (1983a). Texas Star-SR: attenuated *Vibrio cholerae* oral vaccine candidate. *Developments in Biological Standardization* **53**, 59–66.

LEVINE, M.M., KAPER, J.B., BLACK, R.E. AND CLEMENTS, M.L. (1983b). New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. *Microbiological Reviews* **47**, 510–550.

MCALEER, W.J., BUYNAY, E.B., MAIGETTER, R.Z., WAMPLER, D.E., MILLER, W.J. AND HILLEMAN, M.R. (1984). Human hepatitis B vaccine from recombinant yeast. *Nature* **307**, 178–180.

MCKERN, N.M., O'DONNELL, I.J., INGLIS, A.S., STEWART, D.J. AND CLARK, B.L. (1983). Amino acid sequence of pili from *Bacteroides nodosus* (strain 198), the causative organism of ovine footrot. *FEBS Letters* **164**, 149–153.

MAKOFF, A.J., PAYNTER, C.A., ROWLANDS, D.J. AND BOOTHROYD, J.C. (1982). Comparison of the amino acid sequence of the major immunogen from three serotypes of foot-and-mouth disease virus. *Nucleic Acids Research* **10**, 8285–8295.

MAXAM, A.M. AND GILBERT, W. (1977). A new method for sequencing DNA. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 560–564.

MEKALANOS, J.J., SWARTZ, D.J., PEARSON, G.D.N., HARFORD, N., GROYNE, F. AND DE WILDE, M. (1983). Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. *Nature* **306**, 551–556.

MINOR, P.D., SCHILD, G.C., BOOTMAN, J., EVANS, D.M.A., FERGUSON, M., REEVE, P., SPITZ, M., STANWAY, G., CANN, A.J., HAUPTMANN, R., CLARKE, L.D., MOUNTFORD, R.C. AND ALMOND, J.W. (1983). Location and primary structure of a major antigenic site for poliovirus neutralization. *Nature* **301**, 674–679.

MOORE, D.M. (1983). Introduction of a vaccine for foot-and-mouth disease through gene cloning. In *Beltsville Symposia in Agricultural Research, Genetic Engineering in Agriculture, Recombinant Technology May 1982*, pp. 132–142. Allanheld, Osmun, Montclair.

MORIARTY, A.M., HOYER, B.H., SHIH, J., GERIN, W.K. AND HAMER, D. (1981). Expression of the hepatitis B virus surface antigen gene in cell culture by using a simian virus 40 vector. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 2606–2610.

MOSELEY, S.L., HARDY, J.W., IMDADUL HUQ, M., ECHEVERRIA, P. AND FALKOW, S. (1983). Isolation and nucleotide sequence determination of a gene encoding a heat-stable enterotoxin of *Escherichia coli*. *Infection and Immunity* **39**, 1167–1174.

MÜLLER, G.M., SHAPIRA, M. AND ARNON, R. (1982). Anti-influenza response achieved by immunization with a synthetic conjugate. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 569–573.

NEURATH, A.R., KENT, S.B.H. AND STRICK, N. (1982). Specificity of antibodies elicited by a synthetic peptide having a sequence in common with a fragment of a virus protein, the hepatitis B surface antigen. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 7871–7875.

NIMAN, H.L., HOUGHTEN, R.A., WALKER, L.E., REISFELD, R.A., WILSON, I.A., HOGLE, J.M. AND LERNER, R.A. (1983). Generation of protein-reactive antibodies by short peptides is an event of high frequency: implications for the structural basis of immune recognition. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 4949–4953.

PANICALI, D., DAVIS, S.W., WEINBERG, R.L. AND PAOLETTI, E. (1983). Construction of live vaccines by using genetically engineered poxviruses: biological activity of recombinant vaccinia virus expressing influenza virus hemagglutinin. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 5364–5368.

PAOLETTI, E., LIPINSKAS, B.R., SAMSONOFF, C., MERCER, S. AND PANICALI, D. (1984). Construction of live virus vaccines using genetically engineered poxviruses: biological activity of vaccinia virus recombinants expressing the hepatitis B virus surface antigen and the herpes simplex virus glycoprotein D. *Proceedings of the*

HOPP, T.P. AND WOODS, K.R. (1981). Prediction of protein antigenic determinants from amino acid sequences. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 3824–3828.

IVANYI, J., SINHA, S., ASTON, R., CUSSELL, D., KEEN, M. AND SENGUPTA, U. (1983). Definition of species specific and cross-reactive antigenic determinants of *Mycobacterium leprae* using monoclonal antibodies. *Clinical and Experimental Immunology* **52**, 528–536.

KAPER, J.B., LOCKMAN, H., BALDINI, M.M. AND LEVINE, M.M. (1984). Recombinant nontoxinogenic *Vibrio cholerae* strains as attenuated cholera vaccine candidates. *Nature* **308**, 655–658.

KAZIM, A.L. AND ATASSI, M.Z. (1982). Structurally inherent antigenic sites: localisation of the antigenic sites of the alpha-chain of human haemoglobin in 3 host species by a comprehensive synthetic approach. *Biochemical Journal* **203**, 201–208.

KEHOE, M., SELLWOOD, R., SHIPLEY, P. AND DOUGAN, G. (1981). Genetic analysis of K88 mediated adhesion of enterotoxigenic *Escherichia coli*. *Nature* **291**, 122–126.

KEHOE, M., WINTHER, M.D., MORRISSEY, P., DOWD, G. AND DOUGAN, G. (1982). DNA sequence homology between K88 and K99 adhesin antigen determinants of enterotoxigenic *Escherichia coli*. *FEMS Letters* **14**, 129–132.

KEMP, D.J., COPPEL, R.L., COWMAN, A.F., SAINT, R.B., BROWN, G.V. AND ANDERS, R.F. (1983). Expression of *Plasmodium falciparum* blood-stage antigens in *Escherichia coli*: detection with antibodies from immune humans. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 3787–3791.

KLEID, D.G., YANSURA, D., SMALL, B., DOWBENKO, D., MOORE, D.M., GRUBMAN, J.J., MCKERCHER, P.D., MORGAN, D.O., ROBERTSON, B.H. AND BACHRACH, H.L. (1981). Cloned viral protein vaccine for foot-and-mouth disease: responses in cattle and swine. *Science* **214**, 1125–1129.

KLIPSTEIN, F.A., ENGERT, R.F. AND HOUGHTEN, R.A. (1983a). Properties of synthetically produced *Escherichia coli* heat-stable enterotoxin. *Infection and Immunity* **39**, 117–121.

KLIPSTEIN, F.A., ENGERT, R.F. AND HOUGHTEN, R.A. (1983b). Protection in rabbits immunized with a vaccine of *Escherichia coli* heat-stable toxin cross-linked to the heat-labile toxin B subunit. *Infection and Immunity* **40**, 888–893.

KLIPSTEIN, F.A., ENGERT, R.F., CLEMENTS, J.D. AND HOUGHTEN, R.A. (1983). Vaccine for enterotoxigenic *Escherichia coli* based on synthetic heat-stable toxin cross-linked to the B subunit of heat-labile toxin. *Journal of Infectious Diseases* **147**, 318–326.

KÜPPER, H., KELLER, W., KURZ, C.H., FORSS, S., SCHALLER, H., FRANZE, R., STROHMAIER, K., MARQUARDT, O., ZASLAVSKY, V.G. AND HOFSCHNEIDER, H. (1981). Cloning of cDNA of the major antigen of foot-and-mouth disease virus and expression in *E. coli*. *Nature* **289**, 555–559.

LANGBEHEIM, H., ARNON, R. AND SELA, M. (1976). Antiviral effect on MS-2 coliphage obtained with a synthetic antigen. *Proceedings of the National Academy of Sciences of the United States of America* **73**, 4636–4640.

LAPORTE, J., GROSCLAUDE, J., WANTYGHEM, J., BERNARD, S. AND ROUZE, P. (1973). Neutralisation en culture cellulaire du pouvoir infectieux du virus de la fièvre aphteuse par des serums provenant de porcs immunisés à l'aide d'une proteine virale purifiée. *Comptes rendus hebdomadaires des séances de l'Académie des Sciences. Series D* **276**, 3399–3402.

LERNER, R.A. (1982). Tapping the immunological repertoire to produce antibodies of predetermined specificity. *Nature* **299**, 592–596.

LERNER, R.A., GREEN, N., ALEXANDER, H., LIU, F.T., SUTCLIFFE, J.G. AND SHINNICK, T.M. (1981). Chemically synthesized peptides predicted from the nucleotide sequence of the hepatitis B virus genome elicit antibodies reactive with the native envelope protein of Dane particles. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 3403–3407.

LEVINE, M.M., BLACK, R.E., CLEMENTS, M.L., YONG, C.R., LANATA, C., SEARS, S.,

National Academy of Sciences of the United States of America **81**, 193–197.

PARSONS, M., NELSON, R.G. AND AGABIAN, N. (1984). Antigenic variation in African trypanosomes: DNA rearrangements program immune evasion. *Immunology Today* **5**, 43–50.

PERKINS, F.T. (1973). Vaccination against communicable diseases. *Symposia Series in Immunobiological Standardization* **22** 1–407.

PFAFF, E., MUSSGAY, M., BOEHM, H.O., SCHULZ, G.E. AND SCHALLER, H. (1982). Antibodies against a preselected peptide recognise and neutralize foot-and-mouth disease. *EMBO Journal* **1**, 869–874.

PLAYFAIR, J.H.L. (1979). *Immunology at a Glance*. Blackwell Scientific Publications, Oxford.

PRIBNOW, D. (1975). Nucleotide sequence of an RNA polymerase binding site at an early T7 promoter. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 784–788.

RACANIELLO, V.R. AND BALTIMORE, D. (1981). Cloned poliovirus complementary DNA is infectious in mammalian cells. *Science* **214**, 916–919.

ROBERTS, T.M. AND LAUER, G.D. (1979). Maximizing gene expression on a plasmid using recombination *in vitro*. *Methods in Enzymology* **68**, 473–481.

ROBERTSSON, J.A., LINDBERG, A.A., HOISETH, S.K. AND STOCKER, B.A.D. (1983). *Salmonella typhimurium* infection in calves: protection and survival of virulent challenge bacteria after immunization with live or inactivated vaccines. *Infection and Immunity* **41**, 742–750.

ROITT, I.M. (1977). *Essential Immunology*, 3rd Edition. Blackwell Scientific Publications, Oxford.

ROSENBERG, M. AND COURT, D. (1979). Regulatory sequences involved in the promotion and termination of RNA transcription. *Annual Review of Genetics* **13**, 319–353.

RÜTHER, U., KOENEN, M., SIPPEL, A.E. AND MULLER-HILL, B. (1982). Exon cloning: immunoenzymatic identification of exons of the chicken lysozyme gene. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 6852–6855.

SANGER, F., NICKLEN, S. AND COULSON, A.R. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.

SANZEY, B., MERCEREAU, O., TERNYNCK, T. AND KOURILSKY, P. (1976). Methods for identification of recombinants of phage lambda. *Proceedings of the National Academy of Sciences of the United States of America* **73**, 3394–3397.

SCHEID, A. AND CHOPPIN, P.W. (1974). Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis and infectivity by proteolytic cleavage of an inactive precursor protein of sendai virus. *Virology* **57**, 475–490.

SCHERER, G.F.E., WALKINSHAW, M.D., ARNOTT, S. AND MORRÉ, D.J. (1980). The ribosome binding sites recognised by *E. coli* ribosomes have regions with signal character in both the leader and protein coding segments. *Nucleic Acids Research* **8**, 3895–3907.

SHIPLEY, P.L., DOUGAN, G. AND FALKOW, S. (1981). Identification and cloning of the genetic determinant that encodes for the K88ac adherence antigen. *Journal of Bacteriology* **145**, 920–925.

SIGGENS, K., SLOCOMBE, P., EASTON, A., BOSELEY, P., MEAGER, A., TINSLEY, J. AND BURKE, D.(1983). Expression of the human interferon-β gene cloned in phage M13mp7. *Biochimica et biophysica acta* **741**, 65–69.

SKALKA, A. AND SHAPIRO, L. (1976). *In situ* immunoassays for gene translation products in phage plaques and bacterial colonies. *Gene* **1**, 65–79.

SMITH, G.L., MACKETT, M. AND MOSS, B. (1983). Infectious vaccinia virus recombinants that express hepatitis B virus surface antigen. *Nature* **302**, 490–495.

SMITH, H.W. AND LINGGOOD, M.A. (1971). Observations on the pathogenic properties of the K88 Hly and Ent plasmids of *Escherichia coli* with particular reference to

porcine diarrhoea. *Journal of Medical Microbiology* **4**, 467–485.

SNARY, D. (1983). Cell surface glycoproteins of *Trypanosoma cruzi*: protective immunity in mice and antibody levels in human chagasic sera. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **77**, 126–129.

SO, M., DALLAS, W.S. AND FALKOW, S. (1978). Characterization of an *Escherichia coli* plasmid encoding for synthesis of heat-labile toxin: molecular cloning of the toxin determinant. *Infection and Immunity* **21**, 405–411.

STAMM, L.V., KERNER, T.C., BANKAITIS, V.A. AND BASSFORD, P.J. (1983). Identification and preliminary characterization of *Treponema pallidum* protein antigens expressed in *Escherichia coli*. *Infection and Immunity* **41**, 709–721.

STEWART, D.J. (1978). The role of various antigenic fractions of *Bacteroides nodosus* in eliciting protection against footrot in vaccinated sheep. *Research in Veterinary Science* **24**, 14–20.

STOCKER, B.A.D., HOISETH, S.K. AND SMITH, B.P. (1983). Aromatic dependent '*Salmonella* sp.' as live vaccines in mice and calves. *Developments in Biological Standardization* **53**, 47–54.

STROHMAIER, K., FRANZE, R. AND ADAM, K.H. (1982). Location and characterization of the antigenic portion of the FMDV immunizing protein. *Journal of General Virology* **59**, 295–306.

SUTCLIFFE, J.G., SHINNICK, T.M., GREEN, N. AND LERNER, R.A. (1983). Antibodies that react with predetermined sites on proteins. *Science* **219**, 660–666.

VALENZUELA, P., MEDINA, A., RUTTER, W.J., AMMERER, G. AND HALL, B.D. (1982). Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* **298**, 347–350.

VAN DER WERF, S., WYCHOWSKI, C., BRUNEAU, P., BLONDEL, B., CRAINIC, R., HORODNICEANU, F. AND GIRARD, M. (1983). Localization of a poliovirus type 1 neutralization epitope in viral capsid polypeptide VP1. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 5080–5084.

VAN EMDEN, J.D.A., DE GRAAF, F.K., SCHOULS, L.M. AND TEPPEMA, J.S. (1980). Cloning and expression of a deoxyribonucleic acid fragment that encodes for the adhesive antigen K99. *Infection and Immunity* **24**, 1125–1133.

VAN EMDEN, J.D., VAN DER DONK, H.J., VAN EIJK, R.V., VAN DER HEIDE, H.G., DE LONG, J.A., VAN OLDERTON, M.F., OSTERHAUS, A.D. AND SCHOULS, L.E. (1983). Molecular cloning and expression of *Treponema pallidum* DNA in *Escherichia coli* K12. *Infection and Immunity* **42**, 187–196.

VIRJI, M., HECKELS, J.E. AND WATT, P.J. (1983). Monoclonal antibodies to gonococcal pili: studies on antigenic determinants on pili from variants of strain P9. *Journal of General Microbiology* **129**, 1965–1973.

VODKIN, M.H. AND LEPPLA, S.H. (1983). Cloning of the protective antigen gene of *Bacillus anthracis*. *Cell* **34**, 693–697.

WAHDAN, M.H., SERIE, C., CERISIER, Y., SALLAM, S. AND GERMANIER, R. (1982). A controlled field trial of line *Salmonella typhi* strain Ty21a oral vaccine against typhoid: three year results. *Journal of Infectious Diseases* **145**, 292–295.

WALFIELD, A., HANFF, P.A. AND LOVETT, M.A. (1982). Expression of *Treponema pallidum* antigens in *Escherichia coli*. *Science* **216**, 522–523.

WEBSTER, R.G., LAVER, W.G., AIR, G.M. AND SCHILD, G.C. (1982). Molecular mechanisms of variation in influenza viruses. *Nature* **296**, 115–121.

WILLIAMS, J.G. (1981). The preparation and screening of a cDNA clone bank. In *Genetic Engineering* (R. Williamson, Ed.), volume 1, pp. 1–59. Academic Press, New York.

WILSON, I.A., SKEHEL, J.J. AND WILEY, D.C. (1981). Structure of the haemagglutination membrane glycoprotein of influenza virus at 3Å resolution. *Nature* **289**, 366–373.

YOUNG, R.A. AND DAVIS, R.W. (1983). Efficient isolation of genes by using antibody probes. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 1194–1198.