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Biotechnology of Vaccine Development

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Introduction

Vaccination is perhaps the most effective means of controlling infectious diseases. It has been mainly responsible for the eradication of smallpox and for the control of yellow fever, poliomyelitis and German measles in the human population, and of Newcastle disease, foot-and-mouth disease and Marek's disease in domestic animals. This is illustrated clearly by the dramatic decline in the number of cases of diphtheria and polio in Britain (*Figure 1*) and America following the introduction of immunization programmes. A similar dramatic reduction in cases of whooping cough was also evident (*Figure 2*) following the introduction of the pertussis vaccination programme. The recent resurgence of whooping cough notification coincides precisely with the reduction of vaccine uptake due to the alleged side-effect of pertussis immunization.

The art of deliberate immunization against infections has been practised for centuries but the mechanisms of protective immunity were not fully appreciated until the advent of modern immunology. With the discovery of newer technologies and greater understanding of the molecular biology of pathogens, the conventional empirical approaches to vaccine development have given way to more rational design of vaccines. In the past two decades, the

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Abbreviations: ADCC, antibody-dependent cellular cytoxicity; AIDS, acquired immunodeficiency syndrome; BCG, Bacille Calmette-Guerin; BPV, bovine papilloma virus; CMI, cell-mediated immunity; CP$_3$CSS, tripalmitoyl-*S*-glycerylcysteinyl-serine; CRI, cross-reactive idiotypes; DTH, delayed-type hypersensitivity; ETEC, enterotoxigenic *E. coli*; FCA, Freund's complete adjuvant; FMDV, foot-and-mouth disease virus; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; Id, idiotype; IL-2, interleukin-2; i.m., intramuscular; i.p., intraperitoneal; ISCOM, immunostimulating complex; i.v., intravenous; LPS, lipopolysaccharide; LT toxins, heat-labile toxins; MDP, muramyl dipeptide; MHC, major histocompatibility gene complex; OVA, ovalbumin; s.c., subcutaneous; SIV, Simian immunodeficiency virus; ST toxins, heat-stable toxins; Tc, cytotoxic T-cells; TMV, tobacco mosaic virus; TNF, tumour necrosis factor; UDP, uridine diphosphate; VSV, vesicular stomatitis virus.
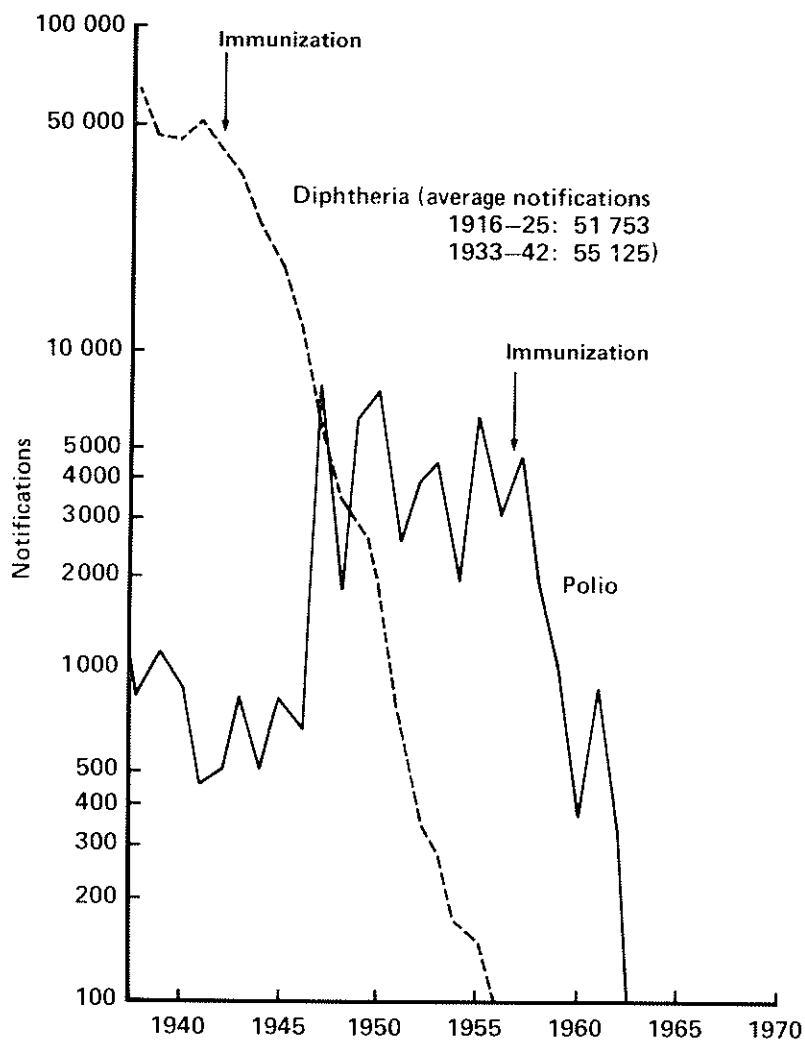

Figure 1. Poliomyelitis (—) and diphtheria (---) notifications, England and Wales, 1940–70 (adapted from Taylor, 1974).

advances of experimental immunology, monoclonal antibodies, recombinant DNA techniques and computer chemistry have influenced greatly approaches to vaccination and, indeed, the whole prospect of controlling some of the most debilitating infectious diseases. Although most of these new strategies are still at the experimental stage, and some will no doubt fall by the wayside, they nevertheless open up a new exciting area of modern medical science with immense potential. This chapter attempts to review some of these novel strategies and evaluate their implications in future immune prophylaxis.
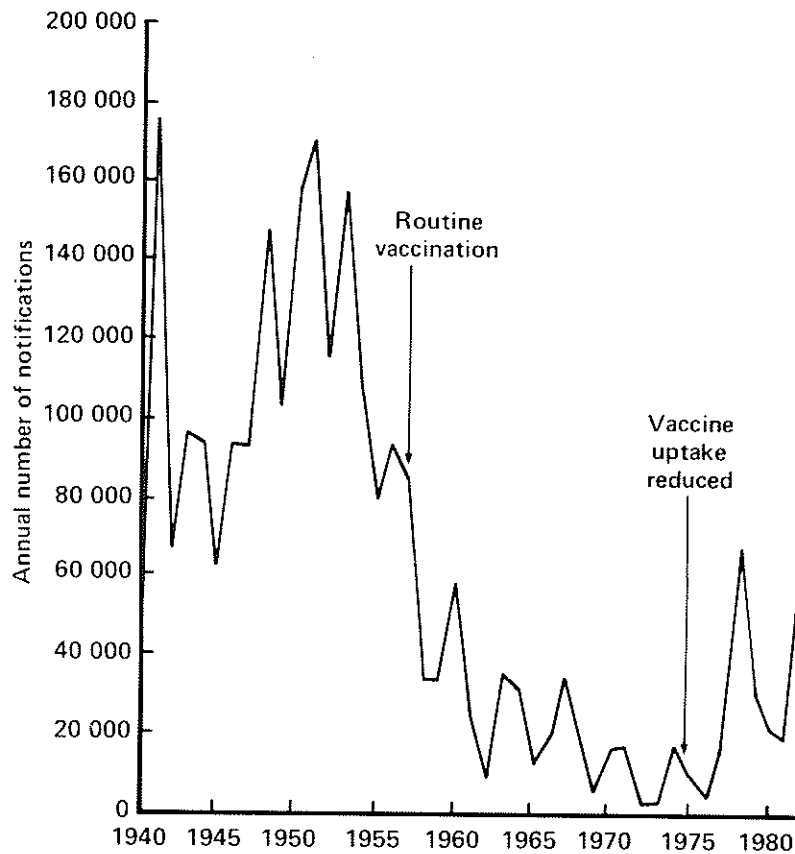
Figure 2. Whooping cough notifications, England and Wales, 1940–82 (from Wells, 1984).

Conventional vaccines

The earliest vaccines were live wild-type organisms. Although these have been mostly replaced by attenuated or killed organisms, some are still in use. For example, viable *Leishmania major* which causes the Old World cutaneous leishmaniasis, or oriental sore, is still used in small doses to induce a controlled lesion at a selected site (*see* Greenblatt, 1980). This practice, which is limited to some endemic areas of high incidence, is far from ideal as it, not surprisingly, incurs too high a risk of developing uncontrolled disease. The risk from viable wild-type vaccines is reduced by attenuation and eliminated by killing by heating or chemical treatment. Attenuation is generally achieved by growing the pathogens in an 'unnatural' host (passage); less commonly viruses have been adapted to grow at a temperature lower than normal (cold-adaptation) or have been rendered temperature sensitive. There are numerous examples of these methods for producing a vaccine. The best known are probably the yellow fever 17D strain developed by passage in mice

and then in chick embryo (Theiler and Smith, 1937), the poliomyelitis strains developed by passaging in monkey kidney cells (Sabin, 1956) and, more recently, passaging of influenza virus strains at lower temperature (Maassab, 1967). For inactivation the pathogens are either subjected to autoclaving or fixed by agents such as formaldehyde, β-propiolactone or, more recently, an imine. Despite the lack of precise scientific basis, these procedures have provided some outstandingly successful vaccines. However, such vaccines may elicit side-effects which are frequently unacceptably harmful to the hosts. Even with the highly successful products, such as the attenuated poliovirus and smallpox vaccines, there is a small but significant number of post-vaccination incidents. Killed vaccines may also present problems in that there is always the chance that some infectious pathogens may survive the inactivation process.

These risks of virulence and contaminants can be reduced somewhat by the use of subunit vaccines, which attempt to enrich the active components by conventional biochemical purification. One of the best examples is provided by vesicular stomatitis virus (VSV), a rhabdovirus that is closely related structurally to rabies virus. It was found that treatment of VSV with ether or a mild detergent resulted in the loss of more than 99.99% of the original infectivity and revealed the presence of spring-like spikeless particles, which showed residual infectivity, and a 70 kDa fraction, which induced neutralizing antibodies (Brown, Cartwright and Small, 1967).

Even in their purest form, these conventional vaccines usually still contain contaminating materials far exceeding, in mass, the immunogenically active ingredients. Such non-essential materials not only cause unpredictable side-effects, they may conceivably counteract and neutralize the induction of protective immunity by the effective antigenic determinants. There is thus a need for a new generation of molecularly defined vaccines which would induce the desirable immune responses capable of controlling particular infectious agents.

The new vaccines

Some of the most profound and far-reaching discoveries in the past decade in biological sciences in general and in immunology in particular are the recombinant DNA techniques (*see* Gilbert and Villa-Komaroff, 1980), monoclonal antibody techniques (Kohler and Milstein, 1975), the idiotype network hypothesis (Jerne, 1974) and the concept of the immune response genes (*see* Moller, 1978). These greatly influence our understanding of the mechanisms of pathogenesis and lay the foundation for new strategies of vaccination against infectious diseases. Current and future vaccine developments based on these findings are discussed in the following sections.

SUBUNIT VACCINES BY RECOMBINANT DNA TECHNIQUE

The constellation of recombinant DNA techniques for placing and maintaining new genetic materials in bacteria, yeasts or mammalian cells is generally known as gene cloning, which is now a powerful tool for synthesizing protein

materials, ranging from peptide hormones and cytokines to subunit vaccines. This exciting technology has been the subject of numerous texts and reviews (Cheung and Kupper, 1983; Smith, Mackett and Moss, 1984; Winther and Dougan, 1984). In principle, the procedure involves finding the gene, insertion of the gene into a plasmid or other suitable carrier, introduction of this complex into bacteria, yeast, flowering plants or mammalian host cells and, finally, the expression and purification of the material desired. In practice, this can be a daunting task, particularly when the protein of interest is available in minute quantities that can only be detected by a tedious bioassay.

Viral vaccines

The overriding impetus for using the recombinant DNA method to produce vaccines is the lack of immunogenic materials. This is certainly the case of hepatitis B virus (HBV) vaccine. Although the purified 22 nm hepatitis B surface antigen (HBsAg) derived from plasma of chronically infected carriers is effective, there is the reservation that the vaccine thus produced may contain other adventitious agents, such as that causing the acquired immunodeficiency syndrome (AIDS). Effective synthesis of HBsAg was achieved in *Escherichia coli* (Burrell *et al.*, 1979) and in the yeast *Saccharomyces cerevisiae* (Valenzuela *et al.*, 1982; Hitzeman *et al.*, 1983; Miyanohara *et al.*, 1983; McAleer *et al.*, 1984; Murray *et al.*, 1984). HBsAg has also been produced in a monkey cell line (COS) infected with a SV40-HVsAg recombinant (Moriarty *et al.*, 1981) and a mouse fibroblast cell line transformed by a bovine papilloma virus-HBsAg (BPV-HBsAg) recombinant (Dubois *et al.*, 1980; Wang *et al.*, 1983). The products from yeast and animal cells resemble those from human plasma, and stimulated antibody synthesis in laboratory animals and induced protection against HBV in chimpanzees (Moriarty *et al.*, 1981; McAleer *et al.*, 1984; Murray *et al.*, 1984) and in humans (McAleer *et al.*, 1984). The yeast-derived recombinant hepatitis B virus vaccine is the first commercially available human vaccine produced by the genetic engineering technology. Its current commercial success has given much encouragement to workers in the field of vaccine research as well as to vaccine manufacturers.

Other subunit viral vaccines are being investigated by recombinant techniques with varying degrees of success. These include the influenza virus haemagglutinin (Emtage *et al.*, 1980; Gething and Sambrook, 1981; Sveda and Lai, 1981), rabies virus glycoproteins (Yelverton *et al.*, 1983), vesicular stomatitis virus (Rose and Shafferman, 1981), herpes simplex virus-1 surface glycoprotein D (Watson *et al.*, 1982), capsid protein VP1 of poliovirus type 1 (van der Werf *et al.*, 1983), avian infectious bronchitis virus glycoprotein (Cavanagh *et al.*, 1984), glycoprotein gp195 of porcine gastroenteritis virus (Hu *et al.*, 1984), envelope and NS1 glycoprotein of dengue virus (Bray *et al.*, 1989) and capsid proteins of hepatitis A virus (Powdrill *et al.*, 1990).

A major current effort in vaccine research is undoubtedly in the field of acquired immunodeficiency syndrome (AIDS). Much has been written in this area and only a brief summary of the latest developments is mentioned here.

The most striking results are the animal model studies with simian immuno-deficiency virus (SIV) by Desrosiers *et al.* (1989) and Murphy-Corb *et al.* (1989). In each case, whole killed SIV were able to delay the onset of disease for a significant time (possibly permanently, as the experiment may eventually demonstrate). Notably, protection against the respective diseases caused by experimental challenge could be achieved even in cases where entry of the virus was not prevented. Thus it may not be necessary to block infection completely in order to have a successful vaccine. Although important in preclinical development, whole-virus AIDS vaccines are not practical against human immunodeficiency virus (HIV) because of the possibility of infectious particles. Thus, intensive efforts are deployed in many laboratories seeking to define the essential components of the virus that would confer effective protection. Along these lines, advances have been made in several directions, including a vast array of neutralizing epitopes, T-cell epitopes that are targets of cytotoxic lymphocytes, and regions of the virus envelope that can target antibodies which mediate antibody-dependent cellular cytotoxicity (ADCC). In total, nearly 20 such sites have been mapped as linear epitopes. The protective mechanisms induced by killed SIV vaccines is at present obscure. It appears that the protection is not directly correlated to specific antibody. In fact, there is *in vitro* evidence that antibody may enhance HIV infection (Bolognesi, 1989). It may be that, in line with other viral infections, CD4 and CD8 cells play the major roles in protection against AIDS. It should also be noted that among all the potential obstacles in the development of an effective AIDS vaccine, by far the most formidable barrier will be the evaluation of efficacy of a candidate vaccine in humans. This is mainly due to the low transmission rate of HIV infection in the population and the long and variable period between infection and disease (Koff and Hoth, 1988). It is hoped that this difficulty may be overcome when more compelling candidate vaccines become available.

Bacterial vaccines

The first commercially available genetically engineered vaccine is a bacterial vaccine effective against enterotoxigenic *E. coli* (ETEC) strains causing diarrhoeal diseases in young piglets. ETEC strains colonize the small intestine of piglets and other animals, including humans, where they secrete enterotoxins which can be classified as heat-labile (LT-toxins) or heat-stable (ST-toxins). The toxins are immunologically conserved. Whilst the ST-toxins of ETEC are small peptides (18 amino acids) and non-immunogenic, the LT-toxins are highly immunogenic. A LT-toxin is composed of two subunits, an enzymatically active A subunit, responsible for the toxic activity of the protein, and an immunogenic B subunit, which is responsible for binding the A subunit to the epithelial cells lining the intestine. The toxin is not purified easily by conventional means and cannot be readily converted to a toxoid. The LT-toxin genes were cloned, the coding sequences within the A subunit gene deleted and a powerful *E. coli* promoter introduced in front of the B subunit gene before transfection into *E. coli* K12, which subsequently

expressed high levels of non-toxic B subunit which may be used as a non-toxic immunogen (So, Dallas and Falkow, 1978; Dallas, Gill and Falkow, 1979). Immunization with the purified B subunit alone induced protective immunity against LT-toxin-producing *E. coli*. A similar strategy may be used to produce recombinant bacterial toxoid vaccines effective against other bacterial diseases.

Current tetanus vaccines are produced by formaldehyde treatment of tetanus toxin of the anaerobic bacterium *Clostridium tetani*. These are effective but induce considerable side-effects. The toxin is a 150 kDa protein of which a moiety, fragment C, comprising the C-terminal 451 amino acids of toxin, has been shown to be non-toxic and yet capable of immunizing mice and guinea-pigs (Helting and Nau, 1984). A polypeptide identical with fragment C has now been expressed in *E. coli*, in a soluble form, permitting its rapid purification (Makoff *et al.*, 1989). This recombinant fragment C showed similar biochemical properties and equal effectiveness in immunizing mice against tetanus as authentic fragment C derived from *C. tetani*. This is likely to be the first of a new generation of tetanus vaccines.

The 69 kDa outer-membrane protein of *Bordetella pertussis* is a fimbrial-membrane non-associated protein that induces agglutinating antibodies in mice. It is detectable in all virulent strains of *B. pertussis*, and an antigenically similar protein is found on the closely related *Bordetella* species. Biochemically purified 69 kDa proteins of *B. pertussis* (Shahin *et al.*, 1990) and *B. bronchiseptica* (Kobisch and Novotny, 1990) have been shown to be protective in mice and piglets, respectively. This protein is also immunogenic in man (Shahin *et al.*, 1990). It has now been cloned and expressed in *E. coli* (Charles *et al.*, 1989) and is undergoing clinical trial. The 69 kDa protein is thus a serious candidate for inclusion in the new formulation of antigenically defined acellular pertussis vaccine.

Parasite vaccines

Recombinant DNA techniques are also being employed to synthesize anti-parasite vaccines, a task which is far more formidable than those of anti-viral or anti-bacterial vaccines. This is due to the extreme complexity of the organisms, coupled with varied life cycles and often poorly understood protective immune mechanisms of the host. It is therefore not surprising that few semblances of a success have emerged. Malaria has received perhaps the most attention because of the prevalent and severe nature of the disease and also because the limited availability of materials excludes the production of conventional vaccines.

Malaria (*Plasmodium* sp.) have a complex life cycle and three different and antigenically distinct stages have been identified: the sporozoite, the infectious form transmitted by the mosquito; the merozoite, the blood stage; and the sexual stage, the gametes. Each of the stages is theoretically susceptible to immunological intervention. At least five groups (Coppel *et al.*, 1984; Dame *et al.*, 1984; Enea *et al.*, 1984; Hall *et al.*, 1984; Koenen *et al.*, 1984) simultaneously reported achieving expression of cloned genes encoding either

sporozoite or blood-stage proteins of the human malaria parasite, *Plasmodium falciparum*. A striking feature of most of these antigens is that they contain short repetitive amino-acid sequences. The significance of these short sequences is at present unclear, but they may well play a role in the generation of antigenic diversity and thus enable the parasite to escape the host immunity. If this genetic diversity and rearrangement is characteristic of the malaria parasite, it may seriously affect the prospect of a recombinant vaccine. To date, none of the antigens expressed is strongly immunogenic and the effectiveness of the protection induced varies greatly with the host–parasite combination used. So far, study in primates has not been outstandingly successful. Three proteins on the surface of sexual-stage *P. falciparum* and *P. gallinaceum* parasites have also been identified as targets of antibodies that block transmission of malaria from host to vector. These antigens provide a basis for developing a transmission-blocking vaccine (reviewed by Miller *et al.*, 1986). Although not directly protective against infection or disease, a transmission-blocking vaccine, combined with a sporozoite or asexual-stage vaccine would reduce the chance of transmission of vaccine-induced mutants. Alone, a transmission-blocking vaccine could reduce transmission below the critical threshold required to maintain an infected population.

It is unlikely that the molecularly defined subunit vaccines produced by recombinant DNA technology will prove to be more effective than conventional inactivated whole organisms which, when available, are in turn generally less efficient than live vaccines. This is largely due to the relative inability of non-replicative organisms to induce effective cell-mediated immunity, such as cytotoxic T-cells or macrophage-activating-specific T-cells which are deemed to be essential for recovery from many infectious diseases. Where this is absolutely the case, an alternative method may have to be used whereby the genes of interest are cloned and transfected into attenuated heterologous carriers.

SUBUNIT VACCINES IN ATTENUATED HETEROLOGOUS CARRIERS

Although a variety of viruses have been used as carriers, they are in general unsuitable because of a restricted capacity for foreign DNA, and are mostly defective, requiring helper virus or special cell lines for replication. Two groups (Mackett, Smith and Moss, 1982; Panicali and Paoletti, 1982), working independently, found that vaccinia virus is unique in that it retains infectivity after accommodating at least 25 000 bp of foreign DNA, which is equivalent to about 20 average genes. This is due to the relatively large size of the viral genome (187 000 bp). In addition, the isolated vaccinia viral DNA is non-infectious and there is evidence that unique transcriptional regulatory sequences are recognizable by the viral DNA polymerase. Furthermore, the successful use of vaccinia virus for the eradication of smallpox suggests that such recombinants, expressing genes of unrelated pathogens, may be used as surrogate live-vaccines.

Basically, a chimeric gene, consisting of the vaccinia virus transcriptional regulatory signal, the thymidine kinase (*tk*) gene and a foreign antigen-coding

sequence is constructed *in vitro* and inserted into a non-essential region of the vaccinia genome by homologous recombination in infected eukaryotic cells. Typically, a special plasmid insertion vector is constructed containing a segment of vaccinia DNA that includes the *tk* gene together with a length of foreign DNA, containing unique restriction endonuclease sites for foreign DNA, which is placed next to the *tk* transcriptional start site. Since the plasmid insertion vectors may contain a variety of unique restriction endonuclease sites, insertion of single or multiple foreign genes can be relatively easily achieved. The next step is to transfect cells that have already been infected with vaccinia virus so that homologous recombination can occur between the *tk* sequences flanking the chimeric gene in the plasmid and the *tk* gene in the vaccinia viral genome. The *tk* recombinant virus is then selected in the presence of 5-bromodeoxyuridine, and isolated plaques are tested for expression of the desired foreign antigen.

Using this system, immunity has been induced in experimental animals against HBV (Moss *et al.*, 1984), influenza virus (Small, Smith and Moss, 1985), herpes simplex virus (Paoletti *et al.*, 1984), VSV (Mackett *et al.*, 1985), respiratory syncytical virus (Elango *et al.*, 1986), rabies virus (Blancuo *et al.*, 1986), polyoma virus (Lathe *et al.*, 1987), Friends murine leukaemia virus (Earl *et al.*, 1986), rinderpest virus (Yilma *et al.*, 1988), malaria (Langford *et al.*, 1986) and HIV (Zagury *et al.*, 1987). In the case of HBV and Friends murine leukaemia virus, the vaccinated animals were protected despite the absence of neutralizing antibody. These animals were immunologically primed and following re-exposure to the foreign antigen upon challenge, mounted an anamnestic immune response that protected against liver disease or erythroleukaemia, respectively. This is analogous to the situation in which mice were protected against herpes simplex virus infection following immunization with purified glycoprotein D, which did not induce neutralizing antibody but activated specific helper T-cells, enabling the animal to mount an accelerated neutralizing antibody response upon challenge infection (Chan, Lukic and Liew, 1985). Protection against polyoma virus-induced tumours was obtained if animals were immunized with vaccinia recombinants expressing polyoma large or middle T-antigens either before exposure to polyoma virus or after polyoma virus-induced tumour had been established (Lathe *et al.*, 1987). In the latter case, vaccination induced tumour regression and complete rejection. Although the mechanism of such an effect is obscure, the finding nevertheless raises the possibility of using vaccinia recombinants as a means of treating established virus-induced cancers, such as those caused by human papilloma viruses.

Although the vaccinia-recombinant system has been reasonably successful in vaccination against various pathogens in experimental animals, there is so far little evidence to demonstrate its efficacy in humans. This absence of data reflects the caution in testing recombinant vaccinia viruses in humans until the foreign genes are inserted into vaccine strains of vaccinia virus and until expression and presentation of the foreign antigens have been optimized.

The use of vaccinia virus as a carrier for human vaccines is likely to be extremely controversial. Vaccinia itself can produce complications, with

adverse reactions occurring at a frequency of one per 1000 (Behbehani, 1983) and with some neurological complications, such as post-vaccinial encephalitis, which are unpredictable and occur in apparently healthy individuals. Research in this area is now concentrating on the specific deletion of genes that are not essential for virus replication in tissue culture but which do decrease virulence in animals (Moss et al., 1988). In addition, insertion of the human interleukin-2 (IL-2) gene into the vaccinia virus also fulfils this expectation (Ramshaw et al., 1987). However, until such time as a suitably attenuated vaccinia mutant, still able to serve as an effective carrier, is obtained, vaccinia virus is unlikely to secure general approval from the licencing authorities as a heterologous carrier, except in the case of extremely serious diseases for which other forms of vaccination are unavailable or unsuitable.

Alternative carriers which are safe and potentially convenient for application are attenuated *Salmonella* strains (reviewed by Winther and Dougan, 1985). In recent years a number of highly stable avirulent mutants of *Salmonella* sp. have been established. Chief among these are the *gal E⁻* mutants (Germanier and Furer, 1971, 1975) and *aro⁻* mutants (Hoiseth and Stocker, 1981). The *gal E⁻* mutants are rough-type strains that are characterized by a block in the enzyme uridine diphosphate (UDP) glucose-4-epimerase (EC 5.1.3.2). The *aro⁻* mutants, on the other hand, have a blockage in the common aromatic biosynthesis (aro) pathway. Both types of mutants retain their ability to penetrate mucosal epithelia and reach the gut-associated lymphoid organs, but fail to replicate in the host tissue in sufficient numbers to cause any pathogenic effect. They have been shown to induce strong protective immunity against wild-type virulent *S. typhimurium* and *S. dublin*. One *gal E⁻* mutant of *S. typhi* (Ty 21a) is now commercially available as a typhoid vaccine. Following these successes, it may be possible to construct strains which protect against more than one enteric pathogen. Formal et al. (1981) were the first to introduce successfully a 129 MDa plasmid from *Shigella sonnei* into Ty 21a. Tests with animals and human volunteers have shown that this vaccine strain is safe even when ingested in high doses (Levine et al., 1983). The gene coding for the *E. coli* heat-labile B-subunit toxin has also been transfected into attenuated *Salmonella* strains with the prospect of producing a bivalent *Salmonella/E. coli* vaccine. It is thus possible that these relatively safe enteric bacteria may serve as effective carriers of oral vaccines for other pathogens, the defence against which necessitates the induction of mucosal and systemic immunity.

Table 1 lists some of the heterologous antigens that have been expressed in *Salmonella* vaccine strains. Thus far, this system has only yielded experimental vaccines. As they stand, they are unsuitable for development as practical live vaccines, which would have to meet rigorous manufacturing standards covering reproducibility of viability and immunogenicity, lack of antibiotic resistance and other safety aspects. A number of approaches could be used to achieve some of these criteria and these are discussed in detail in a recent review (Charles and Dougan, 1990).

Another alternative is to use BCG (Bacille Calmette-Guerin) as an

Table 1. Heterologous antigens expressed in *Salmonella* spp. (adapted from Charles and Dougan, 1990)

Antigen	Plasmid/host	Reference
Form 1 Antigen from *Shigella sonnei*	Native *S. sonnei* plasmid, *S. typhimurium* Ty 21a *gal E* host	Traumont *et al.* (1984)
B-Subunit of the *E. coli* heat labile toxin LT-B	pUC-based plasmid, *S. typhimurium* Ty 21a *gal E* host; pBR322-based plasmid, *S. typhimurium aroA* host	Clements and El-Morshidy (1984); Maskell *et al.* (1987)
K88 antigen from porcine enterotoxigenic *E. coli*	pBR322-based plasmid, *S.typhimurium gal E* host; pBR322-based plasmid, *S. typhimurium aroA* host; *his*-chromosomal vector, *S. typhimurium gal E* host	Stevenson and Manning (1985); Dougan *et al.* (1986); Hone *et al.* (1988)
Schistosoma mansoni antigen	pUC-based plasmid, *S. typhimurium aroA* or *S. typhimurium gal E* host	Taylor *et al.* (1986)
β-Galactosidase	pBR322-based plasmid, *S. typhimurium gal E* host	Brown *et al.* (1987)
E. coli K1 polysaccharide	pBR322-based plasmid, *S. typhimurium aroA* host	O'Callaghan *et al.* (1988)
M5 protein from *Streptococcus pyogenes*	pSC101-based plasmid, *S. typhimurium aroA* host	Poirier, Kehoe and Beachey (1988)
Plasmodium berghei circumsporozoite repeat sequence	pUC-based plasmid, *S. typhimurium* strain WR4017 host	Sadoff *et al.* (1988)
SpaA protein from *Streptococcus sobrinus*	pBR322-based plasmid, *S. typhimurium cya crp* 100 kb virulence-plasmid-deleted host	Curtiss *et al.* (1988)
Infleunza A virus nucleoprotein	pBR322-based plasmid, *S. typhimurium aroA* host	Dougan and Tite (1990)
28 kDa outer membrane protein from *Neisseria meningitidis*	pUC-based plasmid, *S. typhimurium aroA* host	Trakks *et al.* (1989)
Protective 'C' fragment of tetanus toxin of *Clostridium tetani*	pUC-based plasmid, *S. typhimurium aroA* host, *aroC*-chromosomal vector	Strugnell *et al.* (1990)
63 kDa major surface glycoprotein of *Leishmania major*	pBR322-based plasmid, *S. typhimurium aroA* host	Yang *et al.* (1990)

heterologous carrier. BCG, an attenuated bovine tubercule bacillus for vaccination against tuberculosis, is the most widely used live attenuated vaccine in the world. It has been given to over 1.5 billion people with low frequency of serious complications (Bloom, 1989). It requires only a single injection to induce long-lasting (5–50 years) cell-mediated immunity. Furthermore, it is a recognized effective adjuvant, enhancing immune response to many different antigens. The major drawback is that BCG grows very slowly (24 days to produce a colony) and hence is unlikely to be amenable to genetic manipulation. A 'shuttle' strategy has been devised to introduce and express foreign genes into BCG. Basically, phages or plasmids were constructed that can replicate both in *E. coli* and in mycobacteria (Snapper *et al.*, 1988). Foreign genes and selectable markers can be introduced by standard molecular genetic techniques into *E. coli*. The recombinant DNA is then 'shuttled'

into BCG. Foreign genes can then be expressed either by replacing non-essential BCG chromosomal genes or from multicopy plasmids in the cytoplasm. To date, this strategy is still being explored and it remains to be seen whether it will prove to be superior to other methods of vaccination. The great attraction here is the built-in adjuvanticity of the heterologous carrier, BCG.

Attractive as these ideas may be, it must be pointed out that the use of attenuated carriers has certain inherent limitations. Attenuated viruses or bacteria carrying foreign genes and surface antigens could acquire new pathogenicity and tropisms which would require careful examination in each case. In order to eradicate smallpox, a high proportion of the world population was vaccinated with vaccinia virus and will thus have immunity or partial immunity which will, no doubt, limit the replication of vaccinia and thus reduce its effectiveness as carrier. The immunizing effect of the carriers themselves may be a self-limiting factor. For example, even if vaccinia could be used successfully as a carrier for hepatitis B immunization for a defined population, it could not be used again as a carrier for another pathogen in the same population group. To overcome this problem, multivalent vaccines using the same carrier may be constructed. Thus, influenza virus haemagglutinin, hepatitis B virus surface antigen and herpes simplex virus glycoprotein D have been expressed from three distinct loci in a single vaccinia virus recombinant (Paoletti et al., 1985). The recombinants have been shown to elicit significant levels of antibody to the heterologous antigens, and in some cases, protection against disease on subsequent challenge of laboratory animals.

SYNTHETIC PEPTIDE VACCINES

Studies of many globular proteins have concluded that there is an average of only one antigenic determinant per 5 kDa of protein (equivalent to about 40 amino acids). Subunit vaccines, which are macromolecules of various sizes, carry a large number of sites that determine their antigenic specificity. It is now apparent that of these, only a very few are important in provoking protective immunity, whereas others may frequently induce the opposite effect of suppression which could be detrimental to the hosts' defence mechanism. Thus, provided an individual protective determinant can be identified, and isolated in a form retaining its immunogenic properties, it would be possible to produce a highly specific vaccine free from competing and non-essential components. Such peptide vaccines offer several obvious advantages. The product is chemically defined; it is generally stable indefinitely; there can be no inherent infectious agents present, and there is little need for a large-scale production plant. Downstream processing requirements are also absent. Furthermore, because of their stability, they provide the opportunity to use a delayed-release mechanism without loss of the vaccines. In addition, it is possible, at least in theory, to stimulate appropriate immune response by design.

The basis for synthetic peptide vaccines was laid by the pioneering work of

Anderer (1963) who showed that short fragments of the protein from tobacco mosaic virus could inhibit the precipitation of the virus by antiserum, and that a hexapeptide from the fragment, when coupled to bovine serum albumin, induced specific virus precipitating and neutralizing antibodies. Further work by Arnon, Sela and their colleagues (Arnon *et al.*, 1971; Langbeheim, Arnon and Sela, 1976) extended this concept to show that chemically synthesized peptides could also induce antibodies specifically to recognize intact virus particles with the coat-protein from which the amino-acid sequence was derived. With the advent of gene cloning and nucleic acid sequencing techniques, large numbers of amino-acid sequences of biologically important proteins are now available. Undoubtedly, this is responsible for the greatly increased activity in the search for synthetic peptide vaccines by many laboratories (see Lerner, 1982; Brown, 1984; Sela and Arnon, 1984). The first step in developing a synthetic peptide vaccine is to identify the relevant antigen and to determine its amino-acid sequence. This was done originally by laborious chemical sequencing or now, slightly more easily, by deduction from the nucleotide sequence of the gene encoding the protein.

The next step is to identify the relevant antigenic determinants. This is perhaps the most difficult part and may only be achieved by a combination of several methods now available:

1. chemical and enzymatic cleavage of purified proteins and subsequent analysis of their immunological properties;
2. use of monoclonal antibodies to identify and select the smallest components of the antigen which are still capable of specific binding activity;
3. predictions based on regions of hypervariability when the amino-acid sequences of a number of variants are available;
4. predictions of secondary structure by computer chemistry, which indicates regions of hydrophilicity and accessibility based on the state of lowest free energy;
5. predictions based on regions of hydrophilicity and accessibility when the three-dimensional structure is available;
6. random synthesis of overlapping peptides.

So far, no synthetic peptide vaccine has yet reached the stage of clinical or veterinary use. The most promising candidate at the moment is perhaps the synthetic vaccine for foot-and-mouth disease virus (FMDV). From cDNA cloning experiments complete sequences are now available for VP1 from several different serotypes and subtypes of FMDV. There is a 60–72% homology between the nucleic acid sequences for different serotypes and >88% within a single serotype (Makoff *et al.*, 1982; Beck, Feil and Strohmaier, 1983). The variation is concentrated in three discrete regions, residues 41–60, 134–160 and 195–213. Peptides of differing lengths within these regions have been synthesized and their immunogenicity tested. Some of these, particularly those corresponding to residues 134–160, when coupled to a suitable carrier and injected with adjuvant gave rise to virus-neutralizing antibody (Bittle *et al.*, 1982; Pfaff *et al.*, 1982). Moreover, a single inoculation of one of these peptides was sufficient to protect guinea-pigs against subse-

quent infection with the virus. The immunogenicity was only 1–10% of that of the whole inactivated virus, on a weight basis, but was several orders of magnitude greater than that of the whole VP1 protein. This finding provides considerable credence to the notion that synthetic peptide vaccines are not only feasible but that they could be more potent than macromolecular subunit vaccines. However, for commercial acceptance the peptide vaccine will need to have a clear advantage over the present trivalent inactivated FMDV vaccine which is effective and cheap to produce.

As with FMDV, the major immunizing sites of poliovirus appear to be on VP1. The immunogenic region of VP1 was identified by two completely different approaches. Poliovirus type 1 VP1 was expressed in *E. coli* as a bacterial fusion protein which was immunoprecipitated by a virus-neutralizing monoclonal antibody. 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 (van der Werf *et al.*, 1983). This is in excellent agreement with the prediction based on the analysis of antigenic mutants of type 3 poliovirus generated *in vitro* by growing the virus in the presence of neutralizing monoclonal antibodies (Evans *et al.*, 1983; Minor *et al.*, 1983). Several of these mutants had point mutations in the genome which altered the VP1 polypeptide in the region of amino acids 93–100. Five peptides corresponding to residues 11–17, 70–75, 70–80, 93–103 and 97–103 of type 1 VP1 were coupled to carriers and tested for their immunogenicity in rabbits. Only the peptide 93–103 induced neutralizing activity but all five did prime for a long-lasting, virus-neutralizing IgG antibody response following a single inoculation of a small dose of inactivated intact virus (Emini, Jameson and Wimmer, 1983). The results may have important implications in that peptides or other immunogens, although not capable of inducing neutralizing antibody, could nevertheless serve as potent vaccines in that they prime immune memory which could be recalled readily in natural infection.

Influenza virus peptide vaccines have also been investigated with considerable vigour but so far the situation is far from clear. It has been shown that peptides corresponding to regions of the virus haemagglutinin not included in the four major antigenic domains deduced from the three-dimensional structure (Wilson, Skehel and Wiley, 1981) would induce antibodies that react with the intact virus (Muller, Shapira and Arnon, 1982; Sutcliffe *et al.*, 1983). This observation gave rise to the attractive concept that peptides corresponding to a conserved region common to different subtypes of influenza virus may be used as cross-protective vaccines. However, so far, the *in vivo* protective effects, though encouraging, are less than impressive.

Other synthetic peptide vaccines under investigation include cytomegalovirus (Utz *et al.*, 1989), Semliki Forest virus (Grosfeld *et al.*, 1989) and HIV (e.g. Goudsmit *et al.*, 1988). Synthetic peptide vaccines for contraception (Talwar, 1986) and against malaria (Ballou *et al.*, 1987; Herrington *et al.*, 1987; Patarroyo *et al.*, 1988) are currently at various stages of clinical trials, the outcome of which will not be clear for several years.

Clearly, the methods used to predict the immunogenic epitopes would be

different for each peptide vaccine. The following general considerations, however, may be useful:

1. The antigenic sites may be continuous or conformational. The former is determined by the primary sequence of the amino acids, whereas the latter is dependent on the secondary and tertiary structures and is often constructed from discontinuous regions of the protein chain brought into proximity by molecular folding. It is of interest to note that for most globular proteins and native nucleic acids, almost all the antigenic determinants are conformational, whereas for most polysaccharides, fibrillar proteins, such as silk fibroin, and single-stranded nucleic acids, the determinants are sequential (Sela and Arnon, 1984). The conformational epitopes are obviously less readily determined and even more difficult to sythesize. So far all the synthetic peptides tested are based exclusively on contiguous determinants.

2. The minimum size of the peptide chosen is important and should be larger than six amino acids; 15 residues appears to be the norm (Lerner, 1982). Considerably larger peptides have also proved useful but would be difficult to synthesize cheaply on an industrial scale. It is also possible that the larger peptides may assume a fixed conformation distinct from that of native molecules.

3. In selecting an immunogenic peptide, it is essential that at least a part of the sequence be located on the surface of the native molecule so as to be accessible to antibody or immunocompetent cells. For this reason, those regions containing hydrophilic amino acids such as lysine, arginine, aspartic acid and glutamic acid are more likely to be immunogenic. This rule, however, is necessarily imprecise, since some of these amino acids may be involved extensively in interactions with nucleic acid or other viral proteins instead of being available for recognition by the immune system.

4. Peptides that are very hydrophobic are unlikely to be immunogenic. Peptides that are soluble and, in particular, contain proline are better candidates. Proline is important because it occurs at 'corners' which means that the amino acids on either side are near each other, thus producing a minor conformational determinant. In addition, proline residues have an imide rather than an amide bond, so that several atoms surrounding this residue have a three-dimensional relationship to one another and therefore can be recognized by antibody in either situation (Brown, 1984).

5. In general, the main reason for strain variation in the surface antigens of many pathogens is probably the selective pressure of the immune system. Thus, the variable regions of different strains suggest areas available for, and sensitive to, immunological attack. This concept has been exploited particularly successfully in peptide antigens for poliovirus, discussed earlier.

6. In the majority of cases, the immunogenicity of candidate peptide vaccines is screened by their ability to induce neutralizing antibody. Important as it may be, antibody is by no means the only, or even

necessarily the most important, defence mechanism. The T-cell proliferative response should always be included in any assessment of peptide immunogenicity. To this end, the T-cell blot (or T-cell Western blot) developed by Young and Lamb (1986) is of particular interest.

Recently, two prediction methods, AMPHI (Margalit et al., 1987) and Motifs (Rothbard and Taylor, 1988) algorithms have been proposed, based on the hypothesis that immunogenic dominant segments tend to be amphipathic or that there are sequence patterns common to the dominant epitopes. Although both methods have been successful in predicting some epitopes in certain protein molecules (Berzofsky et al., 1987; Cease et al., 1987), the accuracy of prediction for any particular protein molecule is controversial (Gao, Liew and Tite, 1989). It is now generally deemed that they can act only as a rough guide for peptide selection.

In spite of rapid progress in the past few years, synthetic vaccines are still at their early developmental stage. So far, they are confined to peptides with sequential antigenic determinants only. Other types of antigens, such as nucleic acids, polysaccharides or lipids, are currently being explored. Quite apart from identifying immunogenic determinants, other obstacles, such as genetic restriction (i.e. the inability of individuals of certain MHC haplotypes to respond to antigenic stimulation) and a requirement for adjuvants, are yet to be overcome. The former has been demonstrated in several experimental models and clinical systems, whereas the latter necessitates the use of large macromolecules as carriers, hence considerably weakening the advantages attributed to peptide vaccines *per se*. Two recent reports addressed these questions and in one (Francis et al., 1987) it was demonstrated that the genetic restriction could be overcome, at least in the case of FMDV, by linking the peptide to defined helper epitopes from ovalbumin or sperm whole myoglobulin. In the other report (Clarke et al., 1987) it was demonstrated that FMDV peptide (0.2 µg) when expressed with the hepatitis B core protein, elicited levels of neutralizing antibody comparable to those induced by the whole inactivated virus. The antibody protected guinea-pigs against a challenge infection. These observations are likely to have important implications for the future of peptide vaccines in general and FMDV peptide vaccine in particular.

ANTIGENIC MIMICRY USING ANTI-IDIOTYPIC ANTIBODIES

Another novel approach to vaccination is the use of anti-idiotypic antibodies specifically to stimulate the immune response in such a way that the anti-idiotypic antibodies serve as surrogate antigens. So far this is, perhaps, at the least advanced stage of development compared to other previously described strategies.

The region of an antibody molecule that interacts with an antigen is called the *paratope*, which consists of six hypervariable loops, three on the light and three on the heavy chains. These hypervariable areas are made up of varied numbers of determinants, each of which is termed an *idiotope*. The unique collection of idiotopes on each immunoglobulin is named the *idiotype* (Id),

which is defined by the anti-idiotypic antibodies that it can induce. In their pioneering work, Slater, Ward and Kunkel (1955), Kunkel, Mannik and Williams (1963), and Oudin and Michel (1963) independently demonstrated that the idiotypes are specific and unique to individual immunoglobulin molecules. However, later studies with a number of systems revealed that different antibodies may share an idiotype or have similar idiotopes, as recognized by an anti-idiotypic antibody. Idiotypes restricted to a single immunoglobulin are now called *private idiotypes* and those common to more than one immunoglobulin, *public* or *cross-reactive idiotypes* (CRI).

The current interest in using anti-idiotypic antibodies as potential vaccines is based on Jerne's (1974) idiotype network hypothesis (*Figure 3*) which argues the following: if all lymphocytes could recognize virtually all possible antigenic determinants, they should be able to recognize the hypervariable regions on the receptors of other lymphocytes. Idiotypes on one lymphocyte would interact with what would be the anti-idiotype on another. These mutual and ever-spreading idiotypic interactions between lymphocytes would form a vast network spreading through the lymphocyte repertoire of each
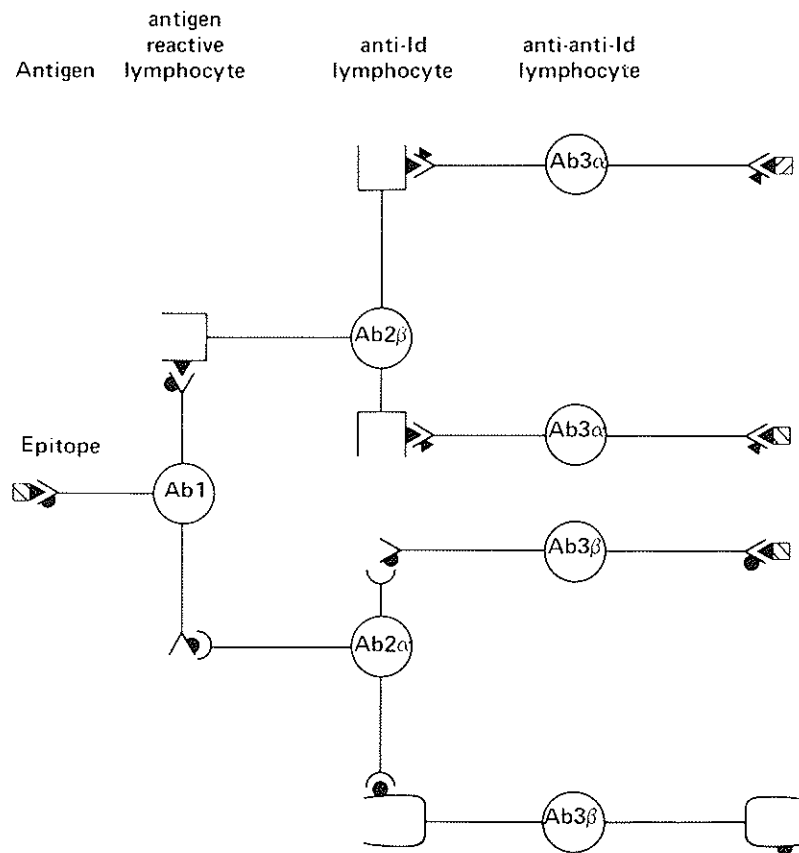

Figure 3. The idiotype network according to Jerne (1974), adapted from Sacks (1985).

individual. The hypothesis further postulates that since both the external antigenic determinants (epitopes) on foreign antigens and the internal idiotopes of a corresponding antibody are each recognized by the same set of paratopes, the epitopes and the idiotopes must be largely overlapping. Therefore, for each foreign antigenic determinant there must be an idiotope which bears its internal image. It is therefore possible to immunize individuals with anti-idiotypic antibody (Ab2α in *Figure 3*) to: (1) perturb the network from its normal equilibrium to favour the desired immune response; and (2) activate specifically antigen-reactive lymphocytes by the internal image (Ab2β).

There is now a considerable body of evidence in support of the validity of the idiotype network hypothesis (reviewed by, for example, Eichmann, 1978). Immunization with anti-Id antibodies (Ab2) has been successful in inducing specific responses to a wide spectrum of foreign antigens, including parasites, bacteria, viruses, alloantigens and tumours (reviewed by, for example, Ivanyi and Moore, 1989). It is clear that, operationally at least, Ab2 antibodies can behave as substitutes for antigens.

The advantages of using anti-Id as vaccines are considerable. As for subunit vaccines produced by gene cloning or peptide vaccines by chemical synthesis, the anti-Id approach would overcome the limitation of antigen source. This approach would also overcome the inherent hazards associated with other forms of vaccines. An additional advantage would be the potential elimination of defective configurational problems which beset antigens produced by gene cloning or synthetic peptides. This approach, when successful, would be monospecific, in that it provides a means of immunizing against single antigenic determinants, uncomplicated by associated, and frequently undesirable, side-reactions. The major disadvantage of an anti-Id vaccine would be its inherent uniqueness.

It seems that Ab2β could be more easily demonstrated if Ab2 prepared from a xenogenic species could induce specific antibody, or better still, Ab2 antibodies could react with conventional specific antibodies from various sources (Nisonoff and Lamoyi, 1981). This has indeed been shown for the following systems:

1. Anti-tobacco mosaic virus (TMV) antibodies were detected in mice using Ab2 prepared against a rabbit idiotype (rabbit Ab1) (Urbain *et al.*, 1984).
2. Hepatitis B surface antigen (HBsAg)-specific antibodies were induced in mice by Ab2 prepared against a common idiotype shared by human antibodies to HBsAg (Kennedy *et al.*, 1983).
3. Neutralizing antibodies against poliovirus type 2 were induced in mice with monoclonal anti-idiotypic antibody (Uytdehaag and Osterhaus, 1985).
4. Stein and Soderstrom (1984) have demonstrated that a monoclonal Ab2 could prime weaning mice for anti-polysaccharide responses and protect against infection with pathogenic *E. coli.*
5. Antibodies specific for the carbohydrate determinant of a *Trypanosoma cruzi* surface glycoprotein (72K) has been induced in BALB/c mice using

a rabbit Ab2 prepared against a mouse monoclonal antibody specific for the same determinant (Sacks, 1985).

The latter two examples are of particular importance since they demonstrated formally that molecular mimicry of a carbohydrate epitope can be achieved. This may be of practical relevance since many microbial antigens contain critical carbohydrate determinants which cannot be reproduced by recombinant DNA technologies.

The genetic restriction may also be circumvented by a B-cell mitogen lipopolysaccharide (LPS). Francotte and Urbain (1984) have reported that, using Ab2, idiotypes that do not recognize conventional antibodies from different species, and therefore do not display the properties of Ab2β, could be induced across a species barrier. In this case, a rabbit Ab2 raised against a 'private' rabbit TMV idiotype, when coupled with LPS, was able to induce anti-TMV antibodies in BALB/c mice. Thus, it appears that the genetic restriction could be overriden by Ab2α, which seems to be able to recognize and activate normally 'silent' idiotypes in the presence of additional mitogenic signals. This finding gives a considerable boost to the prospect of using anti-Id antibody as a potential vaccine. If 'silent' interspecies cross-reaction idiotypes are, indeed, a more general feature of the potential immune repertoire, then their induction of specific immunity by Ab2 in conjunction with additional signals in outbred populations would no longer be dependent upon and limited by the rare occurrence of Ab2β.

Another approach would be to use anti-Id antibodies to activate cell-mediated immunity. Eichmann (1974) originally demonstrated that minute amounts of guinea-pig IgG$_1$ anti-Id injected into mice resulted in the sensitization of both T- and B-cells bearing the recurrent A5A idiotype. Adoptive transfer and *in vitro* culture experiments have demonstrated that anti-Id can sensitize T-cells bearing the relevant Id to provide help for the production of Id-bearing immunoglobulin (Eichmann and Rajewsky, 1975; Cosenza, Julius and Augustin, 1977). These were followed by numerous reports documenting the induction of cytotoxic T-cell response, delayed-type hypersensitivity or suppressor T-cell reactivity by Ab2. It has long been held that T- and B-cells express similar or identical idiotypes. Even though present information suggests no more than 30% homology between T-cell receptor and immunoglobulin (Hendrick *et al.*, 1984; Yanagi *et al.*, 1984), the domain similarity at the V-regions of T- and B-cell receptors may still account for the idiotypic cross-reactivity. A specific T-helper cell clone against sendai virus has been used to raise in syngeneic mice a monoclonal IgM antibody which bound specifically to the T-cell clones. This anti-Id antibody was capable of inducing *in vivo* T-cells which mediated delayed-type hypersensitivity (DTH) and lysed sendai virus-infected target cells across H-2 or allotype barrier (Ertl *et al.*, 1984). In addition, mice pre-immunized with the antibody were protected against a subsequent inoculation of a lethal dose of sendai virus (Ertl and Finberg, 1984).

It thus appears that the anti-idiotypic approach represents an exciting strategy for vaccination. However, to date, reports of success have been few

and far between. This may reflect the considerable difficulty in inducing Ab2 in normally weak immunogenic systems, the syngeneic idiotopes. The general applicability and the strength of the immunity induced by anti-idiotypic antibodies are yet to be fully established.

Immunological considerations

THE PROTECTIVE IMMUNE RESPONSES

The host's specific immunological resistance to infectious organisms can be conveniently divided into humoral and cell-mediated immunity (CMI). These are, however, by no means mutually exclusive, but are often interrelated and frequently need to act in concert for a successful defence network.

Humoral immunity

The importance of humoral antibodies in the host defence against viral bacterial and parasitic diseases has been demonstrated in numerous systems with polyclonal or monoclonal antibodies. There are a number of ways in which specific antibodies could counter an infection: complement-dependent lysis of target cells, direct neutralization, opsonization, antibody-dependent cellular cytotoxicity and immediate-type hypersensitivity.

Specific neutralizing antibodies are perhaps the major defence mechanism against blood-borne pathogens, whether virus, bacteria or protozoa, both at the portal of entry and later clearance from the system. Passively adminis-tered monoclonal antibodies have been shown to be effective in protecting mice against herpes simplex virus (Balachandran, Bacchetti and Rawls, 1982; Chan, 1983), *Neisseria meningitidis* (Moreno *et al.*, 1983), *Plasmodium yeolii* (Freeman, Trejdosiewicz and Cross, 1980) and *Schistosoma mansoni* (Smith *et al.*, 1982). It should be noted, however, that a monoclonal antibody, although by itself not virus-neutralizing nor passively transferring protection, can be used to affinity-purify antigen with strong prophylactic capacity (Chan, Lukic and Liew, 1985). The 110 kDa glycoprotein from herpes simplex virus type 1, for example, although not possessing epitopes for inducing virus-neutralizing antibodies, contains antigenic determinants capable of activating CD4$^+$ helper T-cells. These helper T-cells can adoptively protect mice and enhance neutralizing antibody synthesis upon infectious challenge, probably via a linked-recognition mechanism (Chan, Lukic and Liew, 1985). Local IgA in the respiratory tract has been shown to play an important role in the prevention of influenza virus infection (Liew *et al.*, 1984) and local antibody at the gastrointestinal wall directed against the tissue-penetrating enzymes produced by invading cestodes may be essential for the immunity against *Taenia taeniaeformis* (Kwa and Liew, 1978; Lloyd and Soulsby, 1978).

The facilitation of phagocytosis of infectious agents by macrophages is another important role of antibodies. This operates particularly effectively against blood-borne protozoa such as trypanosomes and parasitized erythro-cytes (Scott and Moyes, 1982). Antibody-dependent cellular cytotoxicity

(ADCC) has been examined with helminth larvae and microfilariae and, in particular, schistosomules of *Schistosoma mansoni* (Jones and Ogilvie, 1971; Butterworth, 1977). The nature of the target antigens, however, is largely unknown. Although ADCC is highly cytotoxic *in vitro* its significance *in vivo* remains to be established. Immediate-type hypersensitivity is principally an IgE-mediated degranulation of mast cells that leads to a hostile local environment for cutaneously (arthropods) and muscosally located (metazoan) parasites. However, the ultimate destruction of parasites probably involves a wide variety of immune responses, including the T-cell-dependent effect of eosinophils, the interaction of eosinophils with the products of mast cell degranulation and the activation of macrophages with IgE immune complexes (reviewed by Mahmoud, 1980).

Cell-mediated immunity

In general, infectious organisms whose primary targets are macrophages should be susceptible to macrophage-activating lymphokines produced by sensitized T-cells. These are T-cells normally causing the manifestation of DTH (Mackaness, 1967; Liew, 1982). Indeed, strong correlations have been found between DTH reactivity and resistance to infection with *Mycobacterium tuberculosis* (von Pirquet, 1909), *Salmonella typhimurium* (Colins and Mackaness, 1968), *Listeria monocytogenes* (Lance and Unanue, 1972), *Mycobacterium leprae* (Godal, 1978) and *Leishmania tropica* (Turk and Bryceson, 1971). However, equally persuasive evidence has also been reported demonstrating a dissociation between acquired cellular resistance to these and other infections and DTH (e.g. reviewed by Hahn and Kaufmann, 1981; Liew, Howard and Hale, 1984). It thus appears that although DTH measures an important facet of macrophage activation by lymphokines, its functional expression is not mandatory for effective intracellular elimination of infectious organisms. In contrast, by arming the host macrophages nonspecifically, the DTH response may produce severe host-tissue damage. This could be particularly deleterious to the host if infections occur in vital organs, as has been demonstrated in the murine influenza model where the transfer of T-cells mediating strong DTH not only failed to protect mice against influenza virus infection (Leung and Ada, 1980) but increased the mortality rate due to influenza pneumonia following challenge with homologous lethal virus (Leung and Ada, 1980; Liew and Russell, 1983). This detrimental effect of DTH can be reversed by the induction of viral-specific suppressor T-cells (Liew and Russell, 1983).

An important recent development is the classification of murine CD4 T-cells into the Th1 and Th2 categories (Mosmann and Coffman, 1987). Early studies showed that T-cells mediating help for antibody synthesis in the carrier–hapten system are distinct from those involved in the DTH to the carrier protein (Liew and Parish, 1974; Silver and Benacerraf, 1974). Furthermore, there appears to be a direct correlation between DTH reactivity and high and low zone tolerance to antibody synthesis (Parish and Liew, 1972). These early findings strongly imply a functional heterogeneity of CD4^{+}

T-cells (Th1 and Th2) providing help to B-cells (Janeway, 1975; Marrack and Kappler, 1975). In leishmaniasis, a protozoan infection, CD4$^+$ T-cells can be either host-protective or disease-promoting (Liew, 1986). However, the heterogeneity of CD4$^+$ T-cells was firmly established only after the discovery that CD4$^+$ T-cells could be subdivided according to the expression of restricted populations of CD45 molecules (the leucocyte common antigens, T200); for recent reviews, *see* Arthur and Mason (1986), Morimoto *et al.* (1986), Thomas and Lefrancois (1988). More importantly, different CD4$^+$ T-cell clones may secrete distinct patterns of lymphokines (Mosmann *et al.*, 1986; Killar *et al.*, 1987). The current consensus on the characteristics and functions of Th1 and Th2 cells in the murine system is shown in *Table 2*.

Table 2.   The lymphokine secretion and function of murine Th1 and Th2 cells

Lymphokines	Th1	Th2
Interleukin-2	+	−
Interferon-γ	+	−
Granulocyte–macrophage colony-stimulating factor	+	+
Interleukin-3	±	+
Interleukin-4	−	+
Interleukin-5	−	+
Functions		
Delayed-type hypersensitivity	+	−
B-cell help	+	+ +
IgE help	−	+
Cytotoxicity	+	+

It should be noted, however, that there is currently some controversy about the existence of such classification in humans because, thus far, most of the available human T-cell clones produce lymphokines that do not fit into the Th1 or Th2 pattern. The reason for such a discrepancy between the murine and the human system is at present unclear; it could well reflect the artificiality of the *in vitro* T-cell cloning system, and the relative stability of human and murine T-cell clones *in vitro*.

In leishmaniasis, an infectious disease model which is extremely amenable to the investigation of cell-mediated immunity, it has been demonstrated recently that Th1 cells predominate in mice which are resistant to and recovering from the disease, whereas Th2 cells are found mainly in mice susceptible to and suffering from the disease (Heinzel *et al.*, 1989). Furthermore, Th1 and Th2 cell lines from mice immunized with *Leishmania* antigens can adoptively transfer host-protection or disease-promotion, respectively (Scott *et al.*, 1988). These findings are of considerable relevance to strategy for vaccination, and underline the importance of prior knowledge of immunological responses to each infectious disease before attempting vaccination.

One of the major developments in contemporary immunology is the extensive study of cytotoxic T (Tc) cells following the discovery of MHC (major histocompatibility gene complex) restriction. That specifically sensitized T-cells could lyse target cells directly had been demonstrated earlier (Hayry and Defendi, 1970). What turns out to be most interesting is that, unlike B-cells, Tc can recognize the target antigens only when the antigens

are presented together with MHC determinants compatible with the Tc cells (Zinkernagel and Doherty, 1974). Subsequently, this has been found to be a general rule for all T-cell subsets. This finding opens up a whole new perspective in viewing T-cell recognition mechanisms and their biological function.

The role of Tc-cells against viral infections has been demonstrated in several systems (reviewed by Zinkernagel and Doherty, 1979; Ada, 1982). However, their effectiveness against bacteria, protozoa and helminths is by no means clear. The efficacy of Tc-cells against infectious agents is based primarily on the destruction of host cells containing replicating pathogens, which when released prematurely could not survive in the host's environment or infect other host cells. This generally works very well against obligatory intracellular infectious agents, such as viruses. However, few protozoan or helminthic parasites have such constraints. It is not surprising, therefore, that despite considerable effort, so far there are very few convincing reports on the presence of Tc-cells in parasitic infections.

Recently, antigen-specific, class I MHC-restricted Tc-cells have been shown to react to *Listeria monocytogenes* (Kaufmann, Hug and De Libero, 1986), *Mycobacterium tuberculosis* (De Libero, Flesch and Kaufmann, 1988), *M. leprae* (Chiplunkar, De Libero and Kaufmann, 1986), *Rickettsia tsutsugamushi/Rickettsia typhi* (Rollwagen, Dasch and Jerrells, 1986) and *Theileria parva* (Morrison, Goddeeris and Teale, 1986). In malaria, it now appears that $CD8^+$ T-cells are essential for host protection (Schofield *et al.*, 1987; Kumar *et al.*, 1988) especially at the sporozoite stage where the role of antibody now seems to be relatively obscure (Hoffman *et al.*, 1987). However, it is not clear whether the protective role of $CD8^+$ T-cells in the bacterial and protozoan infections rests on their cytolytic capacity and/or on the lymphokines they secrete.

General considerations

The following points may be made in considering the type of specific immune response to be aimed at when designing a prospective vaccine:

1. For blood-borne diseases, such as those involving recirculating viruses and haemotoprotozoa, humoral immunity would be important both for the prevention of infection at the site of entry and the subsequent clearance of the pathogen to permit recovery from the disease.
2. Antigens that do not induce neutralizing antibodies yet activate helper T-cells for subsequent enhancement of protective humoral response upon infection, could be as effective as those provoking initial neutralizing antibodies.
3. For obligate intracellular pathogens, macrophage activation by lymphokines secreted by specific T-cells could be the major host protective effector mechanism.
4. However, because of the non-specific nature of the activated macrophages, their stimulation requires careful control to prevent adverse

effects on the host, particularly when vital organs are infected.

5. Cytotoxic T-cell reactivity, although not likely to play a dominant role in prevention of infection, should be important in the recovery from diseases, particularly those involving obligate intracellular pathogens with non-infectious replication stages.

6. Local immunity, such as secretory IgA and immediate-type hypersensitivity, should be very effective against mucosally or cutaneously located infectious organisms.

It should be pointed out that the above list represents the author's personal view and is not intended to be exhaustive or definitive. It underlines, however, the importance of considering each disease as a separate case and devising the vaccination strategy accordingly.

FACTORS DETERMINING THE PREFERENTIAL INDUCTION OF DESIRED IMMUNE RESPONSES

The question of what determines whether a given immune response will occur following a certain regime of antigen administration remains a major enigma of modern immunology. Over the past decade, some answers have begun to emerge, and these may be summarized as follows:

Live v. dead vaccine

It is generally believed that live vaccines are superior to inactivated vaccines in inducing long-lasting protective immunity. This is based on an impressive range of clinical and experimental observations with a variety of diseases. It now seems likely that the superior immunogenicity of live vaccines is not due to the possible higher effective antigenic mass by *in vivo* replication of the organism, but to the efficiency in presentation of the antigens in association with the host MHC determinants on the antigen-presenting cells. Such association has been shown to be mandatory for the effective induction of cell-mediated immunity and, indeed, helper T-cells for humoral antibody responses.

Given this constraint for non-replicative immunogens and the demands for safer and more precisely defined vaccines, the new strategy could be either to introduce the chemically defined antigenic molecules into a safe, clinically acceptable replicative carrier or alternatively, but ideally, to engineer the defined vaccine to mimic the MHC associative requirement. The former approach has been described above (pp. 60–64), and the evidence supporting the feasibility of the latter is summarized below.

In the case of influenza virus infection, the superior immunity induced by live vaccine compared with inactivated or subunit vaccines has been attributed to the relative inability of the latter to activate cytotoxic T-cell precursors into the mature active forms which have been shown to be essential for recovery from infection (McMichael and Askonas, 1978; Yap, Ada and McKenzie, 1978; Lin and Askonas, 1981). Repeated attempts to

induce viral-specific Tc-cells *in vivo* with haemagglutinin, whether as soluble fragments, rosettes, or incorporated into liposomes, have hitherto met with limited success.

The mechanisms by which a given antigen can trigger different subsets of T-cells have been under intense investigation in recent years. This intricate mechanism is not only of great interest to immunologists but is also important in vaccine development. The trend over the past several years has been to group antigens into two categories according to the response they induce (reviewed by Germain, 1986). Exogenous antigens (non-replicating antigens that enter the antigen-presenting cells from outside) are processed in the endosomal compartment and presented at the cell surface in association with class II MHC molecules, to be recognized by and to activate class II restricted $CD4^+$ T-cells. However, endogenous antigens (such as viral proteins that are synthesized inside the cell) are thought to associate with class I MHC molecules to be recognized by and to activate class I restricted $CD8^+$ T-cells. However, recent studies indicate that this rule is not as inflexible as once thought (reviewed by Bolognesi, 1990). Early studies demonstrated that influenza A nucleoprotein, when prepared by the use of a mild detergent (ammonium deoxycholate), stimulates type A virus cross-reactive Tc-cells to a level comparable with that achieved by infectious virus (Wraith and Askonas, 1985). Furthermore, a recombinant DNA-derived hybrid HA_2 molecule of influenza A haemagglutinin, containing a portion of non-structural protein (NS_1), induced virus subtype-specific memory Tc-cells *in vivo* when inoculated into mice with Freund's complete adjuvant (Yamada *et al.*, 1985). More recently, it has been demonstrated that class I Tc can recognize target cells coated with short synthetic peptides corresponding to protein sequences (Townsend *et al.*, 1986). Furthermore, direct introduction of some proteins into the cell cytoplasm results in effective Tc lysis (Moore, Carbone and Bevan, 1988).

Theoretically, if an exogenous peptide can bind to class I MHC molecules on the surface of antigen-presenting cells and subsequently activate Tc *in vitro*, it should be possible to induce a Tc response with the peptide *in vivo*. Carbone and Bevan (1989) successfully primed a Tc response in B6 mice using the synthetic peptide, OVA-229-276 (Ovalbumin). However, OVA-258-276 and peptides of increasing lengths up to OVA-242-276, which were able to sensitize target cells *in vitro*, were inefficient at doing so after intravenous (i.v.) injection. It is argued (Carbone and Bevan, 1989) that the sequence 229–258 in OVA-229-276 provided the necessary signal for the peptide to enter the class I pathway of antigen presentation. Ishioka *et al.* (1989) reported the induction of a Tc response by repeated i.v. immunization of B6 mice with a mixture of peptides derived from trypsin-digested native OVA. Deres *et al.* (1989) also reported that the synthetic peptide (P147–158R⁻) of influenza A virus nucleoprotein covalently linked to tripalmitoyl-*S*-glycerylcysteinyl-serine CP₃CSS) can efficiently prime Tc in BALB/c mice. By contrast, the peptide itself was unable to do so, although it is known that peptide P147–158R⁻, with the deletion of an arginine residue at position 158 of the nucleoprotein, is 1000-fold more efficient than the native

peptide for sensitizing target cells. Takahashi *et al.* (1990) reported that a single subcutaneous immunization in mice with immunostimulating complexes (ISCOMS) containing either purified intact gp160 envelope glycoprotein of the HIV or influenza HA results in reproducible and long-lasting priming of HIV-specific or influenza-specific $CD8^+$, MHC class I restricted Tc-cells. The conclusion from these reports is that although endogenous antigens, in general, are more efficient in inducing class I Tc-cells, exogenous antigens can now also be made to induce these cells. In the case of cutaneous leishmanial infection, it has long been held that only live virulent amastigotes are capable of inducing effective protective immunity, and that visceralizing systemic leishmaniasis is refractory to prophylactic immunization. However, it has been demonstrated that genetically extremely susceptible BALB/c mice, when injected intravenously with lethally irradiated or heat-killed promastigotes of *Leishmania major*, developed strong protective immunity against an otherwise uniformly lethal challenge infection with *L. major* promastigotes and amastigotes (Howard *et al.*, 1982). The precise mechanism for the induction of such protective immunity, which is often as strong as the convalescent immunity, is at present unclear, but is likely to be distinct from that induced by infection, since unlike the latter, this immunization procedure does not induce cutaneous DTH or its memory (Liew *et al.*, 1985).

It thus appears that a more detailed understanding of the immunological events leading to activation of protective immunity would eventually allow the development of procedures which could circumvent, in some cases, the stringent requirement for live vaccines for prophylactic immunization.

Routes of vaccine administration

For mass vaccination, the ideal means of vaccine delivery would be the oral route. However, with few exceptions, until now this route has been the least effective. This is particularly apparent with subunit or peptide vaccines, few of which possess the absorption properties or can withstand the hostile environment of the gastrointestinal tract. The next best means is the percutaneous route: intradermal, subcutaneous and intramuscular. The intraperitoneal and intravenous (i.p. and i.v.) routes, on the other hand, would be impracticable for mass vaccination.

For most viral and bacterial infections, where both humoral and cell-mediated immunities are deemed to be important, percutaneous and i.v./i.p. routes appear equally effective in experimental models (Chanock and Lerner, 1984). In contrast, protective immunity against *Plasmodium berghei* in mice was achieved most potently only via the i.v. routes of immunization with lethally irradiated sporozoites (Cochrane, Nussenzweig and Nardin, 1980) or merozoites (Playfair, De Souza and Cottrell, 1977). This effect of route of infection was also observed in immunizing mice against *Plasmodium yoelii* with affinity-purified glycoproteins (Freeman and Holder, 1983). Similarly, in cutaneous leishmaniasis, protection in susceptible BALB/c mice was only observed following i.v. or i.p. routes of immunization. Irradiated *L. major* promastigotes injected by the subcutaneous (s.c.) or intramuscular (i.m.)

routes were found to be totally ineffective in inducing protective immunity, even when the antigens were administered in conjunction with a range of adjuvants (Howard *et al.*, 1984; Mitchell, Handman and Spithill, 1984). Even more dramatically, s.c. or i.m. injection of killed *L. major* promastigotes, or components thereof, activate a population of $CD4^+$ splenic T-cells which both increases susceptibility and prevents the protective effect of subsequent i.v. immunization (Liew *et al.*, 1985). The precise mechanism or the type of immune response preferentially induced by these various routes is unknown at present but is likely to be involved with the preferential induction of Th2 cells (Liew, 1989). However, if this observation extends to molecularly defined antigens in clinical situations for this and other parasitic infections, it would form a formidable obstacle to mass vaccination.

The mandatory i.v./i.p. administration of vaccines would require a delivery system which mimics these routes. In this case, the carriers such as those described earlier for *Salmonella* mutants by the oral route may be extremely useful. Foreign genes transfected and expressed in these mutants may enable the desired immunogen to be delivered intact, parenterally in a quantitatively controlled manner. This has been achieved recently in an experimental system. Mice immunized orally with an $AroA^-$ mutant (SL3261) of *S. typhimurium* carrying a gene encoding for the major surface glycoprotein (gp63) of *L. major* developed substantial resistance against a challenge infection of *L. major* (Yang *et al.*, 1990).

Alternatively, orally active peptide vaccines substituted at selected residues with unnatural D-isomers (Beddell *et al.*, 1977), which confer a high degree of stability against enzyme degradation, may be constructed to achieve effective absorption and hence mimic i.v. vaccination. This is at least theoretically feasible (Vane and Cuatrecasas, 1984). A realistic example is provided by captopril, an anti-hypertensive agent based on inhibition of angiotensin-converting enzyme. The first such inhibitors were peptides extracted from snake venom (Ferreira, 1965). Knowledge of their structures led to the synthesis (Cushman *et al.*, 1980) of a hybrid molecule which retained the required activity, but was orally active.

Adjuvants

It can be argued that the complex immune mechanism of a mammalian host is the result of evolutionary selective pressure from infectious diseases. As such, human beings have evolved a sophisticated immune system against pathogens which frequently provoke life-long protection after a single or a few infections. This efficient immune recognition network, however, is not likely to cover single or defined antigenic components against which there has been no evolutionary pressure. Hence, it is not surprising that subunit or peptide vaccines are usually only weakly immunogenic and confer little or no protection in the absence of adjuvant.

Experimental studies in animals have revealed an astonishing array of materials which function as adjuvants (reviewed by Jolles and Paraf, 1973; Dukor, Tarcsay and Baschang, 1979; Waksman, 1979; Edelman, 1980).

However, only a few of these are suitable for practical veterinary, and still less clinical use (reviewed by Bomford, 1985; Bomford, 1989). In fact, the only adjuvants in common use in human vaccines are gels of salts of aluminium or calcium which were introduced in the 1920s (Glenny *et al.*, 1926). These adjuvants, although effective for bacterial toxoids, are not adequate for subunit or synthetic peptide vaccines so far tested in experimental systems. For this reason, it is essential that better adjuvants be found. Perhaps even more importantly, we need to understand the mechanism of action of adjuvants. Unfortunately, this has been a generally refractory field of research. So far, a general rule governing adjuvanticity is not discernable.

At present, muramyl dipeptide (MDP) or its derivatives (Ellouz *et al.*, 1974; Chedid *et al.*, 1976) and immunostimulating complex (ISCOM) (Morein *et al.*, 1984) appear to be most promising. These are described briefly here:

1. MDP is a synthetic analogue of a monosaccharide tripeptide isolated from the cell wall of *Mycobacterium* sp. which has proven strong adjuvant activity as a component of Freund's complete adjuvant. A key feature is that MDP not only replaces mycobacteria in oil emulsions, but also works in aqueous solution when administered parenterally or even orally (Audibert *et al.*, 1976) possibly by stimulating macrophages to produce soluble lymphocyte-activating factors (Damais *et al.*, 1982). More importantly, non-pyrogenic derivatives have been synthesized (Chedid *et al.*, 1982) which could bring it closer for clinical use. MDP can also be incorporated into liposomes after the addition of acyl groups or covalently linked to antigens. Such covalent linkage to synthetic peptide antigens has created completely synthetic, chemically defined vaccines with built-in adjuvanticity which have been shown to be as effective as Freund's complete adjuvant (FCA) in experimental systems (Audibert *et al.*, 1982). Recently, a non-pyrogenic threonyl-MDP formulated with squalene-in-water droplets containing pluronic surfactant has also been shown to emulate FCA for the induction of IgG2 antibody and cell-mediated immunity in the guinea-pig (Byars and Alison, 1987).

2. ISCOM is based on the interaction of membrane protein antigens with hydrophobic regions and a matrix of glycoside Quil A extracted from the bark of *Quillaja saponaria* Molina, which forms micelles at the critical micellar concentration of 0·03%. In micelle form, Quil A probably has regions accessible for hydrophobic interaction with the membrane proteins so that it can form complexes with them. ISCOMS proved to be at least 10 times more potent than micelles formed by aggregation of the membrane proteins alone. ISCOMS of parainfluenza-3 (PI-3) and measles viruses also stimulate the formation of antibody to the fusion protein which is considered to be poorly immunogenic (Morein *et al.*, 1984). More importantly, purified HIV-1 envelope protein in ISCOMS was found to be very efficient in inducing strong and long-lasting class I restricted cytotoxic T-cell responses in mice (Takahashi *et al.*, 1990). So far, no side-effects of ISCOMS or of protein micelles have been

observed. Recently, it was reported that interferon-γ could stimulate protective immunity to *P. yoelli* in a mouse model (Playfair and De Souza, 1987) and boosts both the antibody response and CMI. This is the first cytokine to be used as an adjuvant for an anti-parasite vaccine, but others are likely to be tested in the future. Indeed, tumour necrosis factor (TNF) has been found to be able to overcome the disease-exacerbative effect of subcutaneous immunization with soluble antigens of *Leishmania major* and could induce protective immunity when this antigen preparation was injected s.c. together with TNF (Li and Liew, in preparation).

The problems

In spite of past successes and rapid modern technological advances, vaccine manufacturers are currently not in their most confident mood. The number of commercial vaccine producers in the world, and particularly in the United States of America, has declined sharply in the past two decades. In the USA, for most vaccines there are now only one or two suppliers. The reasons for such a state of affairs has been reviewed by Beale (1985) and will be summarized here:

1. The main reason is that vaccines in general are not profitable, particularly when compared to therapeutic drugs. Since most vaccines are used in public programmes or intended for the Third World, there is immense pressure on prices. At least two companies, Lister Institute (now ceased trading) and Wyeth, that played an active part in the WHO campaign for smallpox eradication did not come out of the experience strengthened to partake in another such campaign.
2. Vaccine development is a very expensive and uncertain business. Contrary to popular belief, the major investment in a vaccine occurs not at the research phase but in the developmental stage, i.e. field trials, quality and stability control and large-scale manufacturing. Because the target populations are not always suitable or ethically available, adequate field trials to satisfy the licencing bodies are difficult to conduct. Consequently, limited use and promotion put further pressure on the return from investment.
3. The issue of liability is also an important factor, particularly in the USA. A vaccine manufacturer should rightly be held responsible for any negligence in production but often he is dispiritingly held responsible for accidents which are inherent in the nature of the product or incidents that are temporally but not causally related to vaccination.

These are important practical problems which have to be solved as part of the new strategies for vaccination. There is, fortunately, an increasing awareness by WHO groups and national control authorities about the problems which threaten supply and the need to solve them. There must be commitment to research funding and recognition of the need for adequate profit to the manufacturers to ensure on-going innovation and development by industry. Alternatively, vaccines may be produced by the state institutes, if

vaccine manufacture remains insufficiently profitable. As far as litigation and liability are concerned, governmental compensation systems to assess vaccine damage and to compensate victims of publicly supported immunization programmes could ensure fair and adequate protection for the manufacturer and the victims alike. Finally, the progress towards pure and molecularly defined vaccines would no doubt further minimize the liabilities which bedevilled conventional vaccines based on empiricism.

Table 3. The pattern of progress in vaccine production (adapted from Vane and Cuatrecasas, 1984)

Phases	Vaccines	Relies on
A	Conventional vaccines	Empirical knowledge Living attenuated Crude killed vaccines
B	Improved vaccines	Large-scale culture including mammalian cell culture Purification of antigens Genetic basis for virulence Immunological understanding
C	Single protein vaccines	Monoclonal antibodies Pure antigens Gene cloning and sequencing Improved adjuvants
D	Synthetic peptide vaccines	Immunochemistry Computer chemistry Understanding of basis of adjuvanticity
E	Simple synthetic peptide vaccines active orally	Medicinal chemistry Effective delivery systems

The future

With the advent of new technologies in molecular biology and rapidly expanding knowledge in immunology, there is now excitement about a new upsurge in vaccine development. The progress that may be expected is shown in *Table 3* (Vane and Cuatrecasas, 1984). We are through phases A (conventional vaccine relying on empirical knowledge) and B (improved vaccines based on purification of antigens) and into phase C, using subunit vaccines derived from monoclonal antibodies and gene cloning. The transition from phase C to phase D, synthetic peptide vaccines, has already begun in experimental stages and some are on clinical trial. The final phase, E, based on simple synthetic peptide vaccines active orally, is now theoretically possible.

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